University of Alberta

Branched-chain Amino Acid Requirements in Parenterally and Enterally Fed Neonatal Piglets: Impact of Gut Metabolism

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Spring 2004

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ABSTRACT

Branched-chain amino acids (BCAA; leucine, isoleucine and valine) are nutritionally indispensable amino acids. Their requirements in neonatal piglets have not been experimentally determined. Furthermore, the first step in the catabolism of BCAA is predominantly thought to occur in skeletal muscle, and knowledge of small intestinal catabolism of BCAA is lacking. The BCAA requirements during parenteral and enteral routes of feeding were determined in neonatal piglets using the indicator amino acid oxidation technique. The requirements for total BCAA during parenteral feeding was 56% of enteral feeding, indicating that, during first pass intestinal metabolism, the small intestine utilizes 44% of total BCAA. The ratio of 1:1.8:1.2 (isoleucine/leucine/valine), found in most foods, was subsequently determined to be appropriate during enteral feeding. However, during parenteral feeding, isoleucine was first limiting and valine was second limiting for protein synthesis. Thus, route of feeding has a considerable influence on BCAA metabolism, suggesting that the gut either converts BCAA into their respective ketoacids, utilizes BCAA for protein synthesis or catabolizes to CO_2 for energy.

To determine whether enterocytes (small intestinal epithelial cells) could completely catabolize BCAA, procedures were developed to isolate enterocytes, examine BCAA catabolizing enzyme activity and conduct in-vitro BCAA oxidation studies. Adult porcine enterocytes possessed branched-chain aminotransferase activity (20% of muscle) and were shown to completely catabolize BCAA to CO_2. The highest oxidation values were observed for leucine and ketoisocaproic acid. In the final study, neonatal porcine enterocytes were isolated from 0, 3 and 7 day old piglets to examine catabolism of BCAA

during the early stages of growth. Catabolism of BCAA to CO_2, was observed in neonatal enterocytes, although no influence of age was observed. These studies clearly show that the gut has a considerable capacity to catabolize BCAA to CO_2, and are providing significant fuel to the rapidly growing small intestine in the neonate.

To my wife, Gayathri

Who has been a constant source of support, love and courage over these many years.

Truly, you are a blessing to me.

Acknowledgements

My first and most sincere thanks to my supervisor, Dr. Ron Ball who gave me the opportunity to work with him. Over the years he has guided and advised me to achieve not only scientific, but personal goals as well. Dr. Paul Pencharz, my co-supervisor has been a great teacher and I will always be thankful for his advice and support. They both have been amazing mentors these past years, and taught me to think and analyze critically, and also allowed me to grow personally. For this, I will be forever thankful.

I thank Dr. Tapan Basu, my supervisory committee member for always being there. His office has always been open for me.

Dr. Vickie Baracos has been a source of amazing discussions. I have never met a person who could challenge an individual, yet at the same time be fair. Thank you for being the person who you are and my life has definitely been enriched because of you.

I would like to thank Dr. Douglas Burrin, my external examiner, whom I have known for the entire course of my program through various conference meetings. Your thoughts and inputs into my research are well acknowledged.

I would like to especially thank two people who have become good friends and colleagues, Desmond Pink and Kate Shoveller. Kate, for all the help that you provided during the early part of my program will never be forgotten. Des, for all the endless days of biochemical experiments that we endured. Over the years, they both have become my constant source of support, friendship and 'partners in crime'.

During the entire course of my program various other people have helped me and I thank them all; Suzan Cvitkovic, Garson Law, Robyn Harte, Jakob Attakora, Vera Mazurak, Michelle Mackenzie and countless others.

My special thanks to Research Associates Drs. Soenke Moehn, Janet Brunton and Rob Bertolo, for assistance with laboratory methods and making sure that my experiments ran smoothly.

I would also like to take this opportunity to thank all administration staff at the Department of Agricultural, Food and Nutritional Science, especially Ms. Jody Forslund. All staff at the Metabolic Unit, SRTC and Biotech Centre, especially Ms. Renate Meuser, and Ms. Joan Turchinsky.

I will forever be indebted to my loving parents, who gave me the courage, love and encouragement to go out and achieve my goals. I will always love them for believing in me and what I can achieve.

Lastly and most importantly I would like to thank my beloved wife, Gayathri, who has stood by me during times of hardship and happiness alike. This work could not have come to a completion without your love and faith in me.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

The data presented in Chapters 3.0, and 4.0 have been previously published in peer-reviewed journals:

Elango, R., Pencharz, P.B. & Ball, R.O. (2002) **The branched-chain amino acid requirement of parenterally fed neonatal piglets is less than the enteral requirement.** J. Nutr. 132: 3123-3129.

Elango, R., Goonewardene. L.A., Pencharz, P.B. & Ball, R.O. (2004) **Parenteral and enteral routes of feeding in neonatal piglets require different ratios of branched-chain amino acids.** J. Nutr. 134: 72-78.

Portions of research from this thesis have been presented as abstracts:

Elango, R., D Pink, PB Pencharz & RO Ball. **Branched chain acid catabolism in the neonatal piglet gut.** 9th International Symposium on Digestive Physiology in Pigs. Banff, Alberta , May 2003, Vol.2, pg 207 (Poster Presentation)

Elango, R., D Pink, PB Pencharz & RO Ball. **Branched-chain amino acid catabolism during postnatal stages of growth and development in the neonatal piglet,** ABS # 431.1, Experimental Biology, San Diego, California, April 2003. (Oral Presentation)

Elango, R., D Pink, PB Pencharz & RO Ball. **Branched chain amino transferase (BCAT) activity exists in isolated porcine enterocytes.** ABS # 014, pg.80. Proceedings of the Canadian Federation of Biological Societies, Montreal, Quebec. June, 2002. (Poster Presentation)

Elango, R., PB Pencharz & RO Ball. **The optimum ratio of branched chain amino acid differs between enteral and parenteral routes of feeding.** ABS # 567.6 Experimental Biology, New Orleans, Louisiana, April 2002. (Oral Presentation)

Elango, R., PB Pencharz & RO Ball. **The neonatal piglet small intestine utilizes 44% of Total branched chain amino acids (BCAA) delivered enterally.** ABS # 029 Canadian Federation of Biological Societies, Ottawa, Ontario. June, 2001. (Oral and Poster Presentation)

Elango, R., PB Pencharz, RO Ball. **The parenteral requirement for branched chain amino acids (BCAA) in the neonatal piglet is 56% of the enteral requirement.** ABS # 328.5 Experimental Biology, Orlando, Florida, March 2001. (Oral Presentation)

Elango, R., PB Pencharz & RO Ball. **A multi-catheter piglet model for determining amino acid requirements and metabolism in the neonate.** ABS # 109, pg. 63 Proceedings of the Canadian Federation of Biological Societies, Winnipeg, Manitoba, June 1999. (Poster Presentation)

Extension Publications

Elango, R., D Pink, PB Pencharz & RO Ball. Small intestinal utilization of branched chain amino acids. Advances in Pork Production 14: A13, 2003

Elango, R, Shoveller, AK, Pencharz, PB & Ball, RO. Ideal ratio of branched chain amino acids (BCAA) in early-weaned piglets Advances in Pork Production 13: A37, 2002.

Elango, R Pencharz, PB & Ball, RO. Branched chain amino acid requirements of the newborn piglet. Advances in Pork Production 12: A26, 2001.

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LIST OF ABBREVIATIONS

ALAT, alanine aminotransferase

BCAA, branched-chain amino acids

BCAT, branched chain aminotransferase

BCDH, branched-chain dehydrogenase

BCKA, branched chain alpha-ketoacids

BCA, bicinchoninic acid

BSA, bovine serum albumin

CI, confidence interval

DTT, dithiothreitol

HPLC, high performance liquid chromatography

IAAO, indicator amino acid oxidation

IDAA, indispensable amino acids

KHB, Krebs Henseleit Bicarbonate

KIC, ketoisocaproic acid

KIV, ketoisovaleric acid

KMV, ketomethylvaleric acid

LNAA, large neutral amino acids

LBW, low birth weight infants

LDH, lactate dehydrogenase

PBS, phosphate buffered saline

SRA, specific radioactivity

TPN, total parenteral nutrition

1.0 INTRODUCTION

Low birth weight infants (LBW), infants less than 2500g at birth, constitute a majority of infants born prematurely (<37 weeks gestation). In the United States, 1999 statistics reveal 7.6% of infants were LBW of the 11.8% preterm infant births. Mortality rates are increased with LBW infants and could be 65% of all infant births (Klein, 2002). LBW infants are often unable to tolerate oral feedings due to metabolic and gastrointestinal immaturity, and a variety of associated factors such as short bowel syndrome, chronic diarrhea and respiratory problems (Ball et al, 1996). Thus neonatal nutrition support strategy involves parenteral route of nutrient delivery until enteral feedings can be tolerated. The goal of any nutrition support is to provide balanced nutrients to support adequate growth of the neonate without compromising the immature metabolic system. In spite of advances in parenteral nutrition support, achieving energy and protein accretion rates in preterm neonates to that seen in the normally growing fetus of the same gestational age, has not been possible (Thureen & Hay, 2001).

A supply of the "optimal" balance of indispensable and dispensable amino acids is necessary to maintain growth and body protein deposition. Protein synthesis and growth are limited by the first limiting amino acid in the dietary supply. Furthermore in parenterally fed neonates the risk of supplying excess of an amino acid is also possible due to the immaturity of the neonate's metabolic system. Currently available parenteral formulas do not supply the appropriate balance of amino acids (Brunton et al, 2000). Thus, directly determining the parenteral requirement for dispensable and conditionally dispensable amino acids in neonatal populations is critical.

Initiation of early enteral nutrition following parenteral feeding is often recommended in clinical practice to aid normal gastrointestinal growth and decrease parenteral nutrition associated cholestasis. Furthermore, prolonged parenteral nutrition has been shown to cause gut atrophy due to lack of luminal nutrients. The small intestine is now recognized as an organ where significant degradation and synthesis of nutrients takes place, apart from its role in digestion and absorption. Less data are available concerning differences in metabolism due to parenteral versus enteral delivery. Thus comparison of amino acid requirements during parenteral versus enteral nutrition should provide important information with respect to metabolism of amino acids during first pass intestinal metabolism versus the complete lack thereof.

The review of literature presented in this thesis will focus on parenteral nutrition, amino acid requirements during parenteral nutrition and the effects of parenteral versus enteral nutrition. The neonatal piglet as a model for human infants will also be discussed. Catabolism of branched-chain amino acids, antagonism among BCAA, and inter-organ differences in BCAA catabolism will then be discussed. Finally, since BCAA enriched nutrition support is quite common in clinical settings, the various conditions under which BCAA supplementation occurs will be discussed.

1.1 Total Parenteral Nutrition

Total parenteral nutrition (TPN) refers to the complete supply of nutrients required by the body for its metabolic needs through a central or peripheral vein bypassing the gut (Cataldo et al, 1998). Parenteral nutrition (PN) is referred to as total or supplementary, to designate amount and also referred to as central or peripheral to

indicate route (Shills et al, 1994). Supplementary parenteral nutrition is advised or applied in conditions where the neonate is able to tolerate some nutrients enterally. Parenteral feeding is used in this case only to supplement the missing nutrients or calories.

1.1.1 Amino Acid Requirements during TPN

Parenteral route of feeding by passes the gastrointestinal tract and significantly affects whole body metabolism and especially nitrogen and protein metabolism (Duffy & Pencharz, 1986). Amino acids are provided to the peripheral circulation in concentrations which are normally not observed during enteral nutrition due to first pass intestinal metabolism. Bertolo et al (2000) observed significant changes in organ and plasma amino acid concentrations when neonatal piglets were fed identical diets via intragastric, intraportal or intravenous route. In similar neonatal piglets, Dudley et al (1998) observed that during parenteral nutrition, fractional synthesis rates of mucosal proteins were lower due to route of feeding. Indeed, the requirements vary significantly between the routes of feeding for some of the amino acids tested thus far. Threonine (Bertolo et al, 1998), methionine (Shoveller et al, 2003), phenylalanine (House et al, 1997) and lysine (House et al, 1998) requirements have been shown to be lower in parenteral than enteral nutrition. Parenteral threonine was 60%, methionine 31% and phenylalanine and lysine were 30% of the enteral requirement. Tryptophan requirements were shown to be similar between routes of feeding (Cvitkovic et al, 2004). The decreased parenteral requirements clearly indicate that indispensable amino acids being supplied in current parenteral feedings are in excess and could potentially endanger

neonatal health. The requirements for branched-chain amino acids; isoleucine, leucine and valine during parenteral nutrition is yet to be determined directly. Whether their requirements vary due to route of feeding is unknown and needs to be examined.

1.1.2 TPN versus Enteral nutrition

The significant differences among amino acid requirements due to parenteral feeding indicates that the small intestinal utilization of indispensable amino acids is more than that required for intestinal protein synthesis alone. The various fates of the extracted amino acids are under investigation currently (Wu 1998). Stoll et al (1998) in orally milk-fed piglets conducted mass balance studies across the portal drained viscera and observed that during first pass metabolism, utilization of indispensable amino acids were significant and varied among the amino acids. 62% of threonine, 46% of lysine, 40% of phenylalanine, 52% of methionine, 31% of isoleucine, 39% of valine and 43% of leucine were reported to be taken up by the portal drained viscera. Subsequently, van Goudoever et al (2000) showed in neonatal piglets that lysine is catabolized by the intestines and accounts for 31% of whole body lysine oxidation. Yu et al (1990) in enterally fed dogs showed that leucine oxidation by the splanchnic organs was 30-35% of ingested leucine. Thus, during enteral feeding indispensable amino acids are metabolized to a greater extent than appreciated previously. This suggests that in neonatal populations with a rapidly growing and developing gut that, the enteral requirement for these amino acids could be higher than that previously understood and therefore needs to be directly determined.

1.1.3 The Piglet as a Model for the Preterm Neonate

Practical and ethical limitations exist when studying metabolism of amino acids in human neonates. With respect to requirement studies, it is unethical to maintain neonates on amino acid deficient diets for prolonged periods of time; therefore an animal model is required. The neonatal piglet has been used successfully as a model for examining nutritional problems and discussed extensively (Benevenga 1986, Miller & Ullrey 1987, Moughan et al 1992, Shulman 1993, Ball et al 1996). This is because various similarities exist between neonates and piglets in organ systems, body compositions, pattern of gastrointestinal growth and haematologic parameters (Ball et al, 1996). Specifically, with respect to amino acids and protein synthesis, the similarities are closer. Plasma and milk amino acid patterns in both species are similar. Furthermore, existing estimates of amino acid requirements between the two species are similar (g/100g protein) and therefore the results obtained from requirement studies matches closely with human neonates. At birth, the piglet is relatively immature exhibiting poor thermoregulatory capacity, low birth weight, low fat reserves and high metabolic and protein synthetic rate (Ball et al, 1996). Thus, the neonatal piglet is a good model for premature infants. From a practical point, piglets allow ease of handling and housing, are comparatively inexpensive, are a more homogenous population and experiments can be designed to be more invasive to collect blood and tissue samples. Furthermore, in the swine industry the practice of segregated early weaning has necessitated research into weaning-induced gut stress and related problems. Thus, results from neonatal TPN piglet studies have implications in both human infants and the swine industry.

1.2 Branched-chain Amino Acids

The branched-chain amino acids (BCAA), leucine, isoleucine and valine cannot be synthesized by mammals, and are therefore essential nutrients that must be obtained from dietary sources (Wagenmakers and Soeters, 1995). The BCAA are quantitatively by far the largest single group among the dietary indispensable amino acids. They comprise about 35% of the indispensable amino acids in muscle proteins and about 40% of the indispensable amino acid requirements in mammals (Schadewaldt and Wendel, 1997). The principal roles of BCAA include: tissue protein synthesis; carbon precursors for synthesis of tricarboxylic acid (TCA) cycle intermediates, ketone bodies and fat; carbon and nitrogen precursors for synthesis of alanine, glutamate and glutamine; and energy source via oxidation to CO_2 (Harper et al, 1984).

1.2.1 Catabolism of BCAA

The catabolic pathways of the three BCAA have several features in common. The initial step for each is transamination, a readily reversible reaction that yields the corresponding branched-chain ketoacid (BCKA). (**Figure 1.1**). This reaction is catalyzed by branched-chain aminotransferase [BCAT, EC 2.6.1.42] (DeSantiago et al, 1998). Each BCKA then undergoes irreversible oxidative decarboxylation, the product of which is the acyl Co-A derivative with one less carbon. This is catalyzed by a mitochondrial enzyme complex, branched-chain α - ketoacid dehydrogenase [BCDH - EC 1.2.4.4] (DeSantiago et al, 1998). Thereafter the pathways

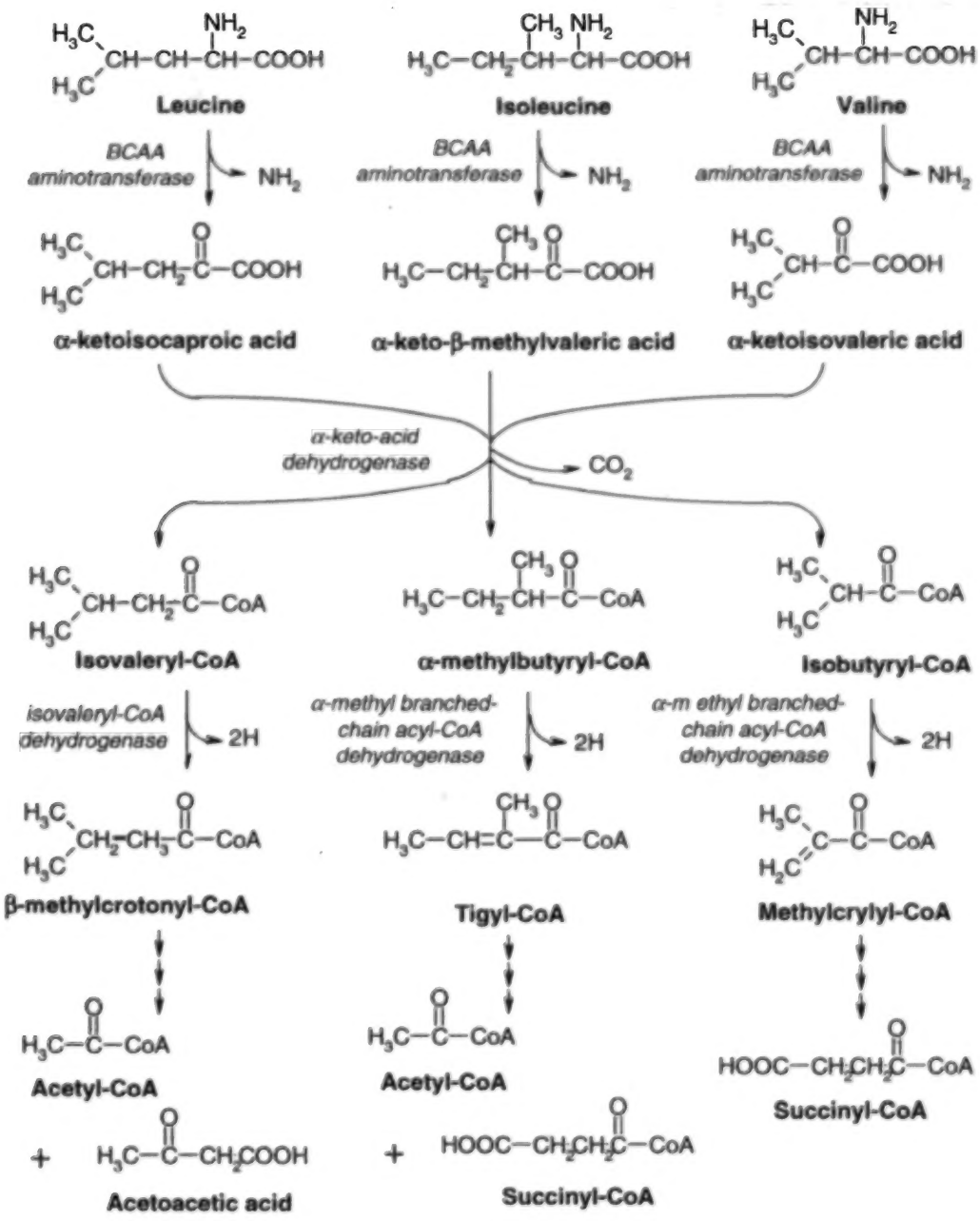

Figure 1.1 Branched-chain amino acid catabolism in mammals

resemble those for fatty acid oxidation and lead to end products that can enter the TCA cycle (Harper et al, 1984). The end products of isoleucine catabolism are propionyl-CoA and acetyl-CoA, and hence is both glucogenic and ketogenic. Leucine catabolism yields acetoacetate and acetyl-CoA, and therefore is ketogenic. Valine catabolism yields succinyl-CoA, and therefore is glucogenic (Harper et al, 1984).

1.2.1.1 Branched-chain Aminotransferase

The aminotransferase is widely distributed in most mammalian tissues. Branched-chain amino transferase (BCAT) is found in mammals in both mitochondrial, (BCATmitochondrial, BCATm) and cytosolic, (BCATcytosolic, BCATc) compartments (DeSantiago et al, 1998, Yudkoff, 1997). In most tissues, like heart, skeletal muscle, stomach, pancreas and kidney the mitochondrial form predominates. The cytosolic form is present in brain, ovary and placenta (Sweatt et al, 2003). Tissue distribution of BCATm activity differs significantly between species and among various organs (**Table 1.1**). The most striking difference is observed in rats, where pancreas, stomach, heart and skeletal muscle have significantly high BCAT activity, and rat liver has almost negligible BCAT activity (Table 1.1). Human tissues have a more uniform activity spread across tissues for the aminotransferase.

In mammalian tissues, three different isoforms of the BCAT have been identified. Enzyme I, which accepts all three BCAA as substrates, is the widely prevalent form in cytosol and mitochondria of most tissues (Ichihara, 1975). Enzyme II is leucine specific and has been reported in rat liver cytosol. Enzyme III is mostly found in pig and heart brain. A leucine and methionine specific aminotransferase in rat liver mitochondria

Table 1.1 Distribution of branched-chain aminotransferase (BCAT)[1] activity in rat, human and monkey tissues

Tissue	Rat[2]	Human[3]	Monkey[4]
	mU/g wet wt		
Heart	4894 ± 216	387 ± 23	NA
Muscle	1599 ± 60	124 ± 14	245 ± 29
Brain	1944 ± 94	510 ± 49	434 ± 39
Liver	78 ± 5	248 ± 32	250 ± 29
Kidney	3486 ± 142	880 ± 48	1215 ± 133
Pancreas	11088 ± 1187	NA	1790 ± 185
Stomach	5842 ± 415	447	559 ± 57
Small intestine	489 ± 22	241 ± 11	383 ± 22
Colon	894 ± 37	254 ± 23	NA
Adipose	166 ± 16	84 ± 4	98 ± 10

Note: Values are mean ± SEM; NA, not available

[1] BCAT activity measured by quantitative extraction from homogenized tissue samples

[2] Rats weighing 250-300g

[3] Patients undergoing surgical procedures, 2 mo to 68 yrs

[4] African green monkeys aged 4-5yrs, weighing ~4kg

Source: Suryawan et al (1998)

has also been reported, although its physiological role is yet to be determined (Harper et al, 1984).

BCAT enzyme activity has no known regulation. Thus, the rate of transamination is dependent on concentrations of the enzyme and substrates. BCAT has a relatively higher Michaelis-Menten constants (K_m) for all 3 BCAA, compared to the second enzyme in BCAA catabolism, BCDH. In particular, BCAT activity has a higher K_m for valine than isoleucine and leucine, which possibly explains the higher plasma valine concentrations observed in most species when compared to isoleucine and leucine (**Table 1.2**).

1.2.1.2 Branched-chain Dehydrogenase

Branched-chain dehydrogenase (BCDH) enzyme catalyzes the second and committed step in BCAA degradation, and results in the decarboxylation of the first carbon skeleton. The BCDH complex is very similar to the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complex, which are also housed in the matrix of the inner mitochondrial membrane. The BCDH complex contains three catalytic component enzymes, a branched-chain α-ketoacid decarboxylase (E1) composed of 2α and 2β subunits, a dihydrolipoyl transacylase (E2) and a dihydrolipoyl dehydrogenase (E3) (Chuang & Shih, 1995). The combined catalytic action of the 3 components in the presence of thiamine-pyrophosphate, FAD, NAD and CoA produces the branched-chain acyl CoA, CO_2 and NADH from the respective BCKA (Figure 1.1 and **Figure 1.2**).

Table 1.2 Michaelis-Menten Constants (K_m) for branched-chain amino acid (BCAA) catabolizing enzymes[1]

Amino Acid	BCAT[2]	Keto Acid	BCDH[3]	KIC di[4]
	K_m (mM)		K_m (μM)	K_m (μM)
Isoleucine	0.8	KMV	14	-
Leucine	0.4 – 0.8	KIC	15	0.32
Valine	1.2 – 4.3	KIV	28	-

[1]Source: Chuang and Shih (1995)

[2] From rat tissues for BCAT enzyme I and III

[3] From rabbit tissues

[4] KIC di-oxygenase; cytosolic liver and kidney enzyme

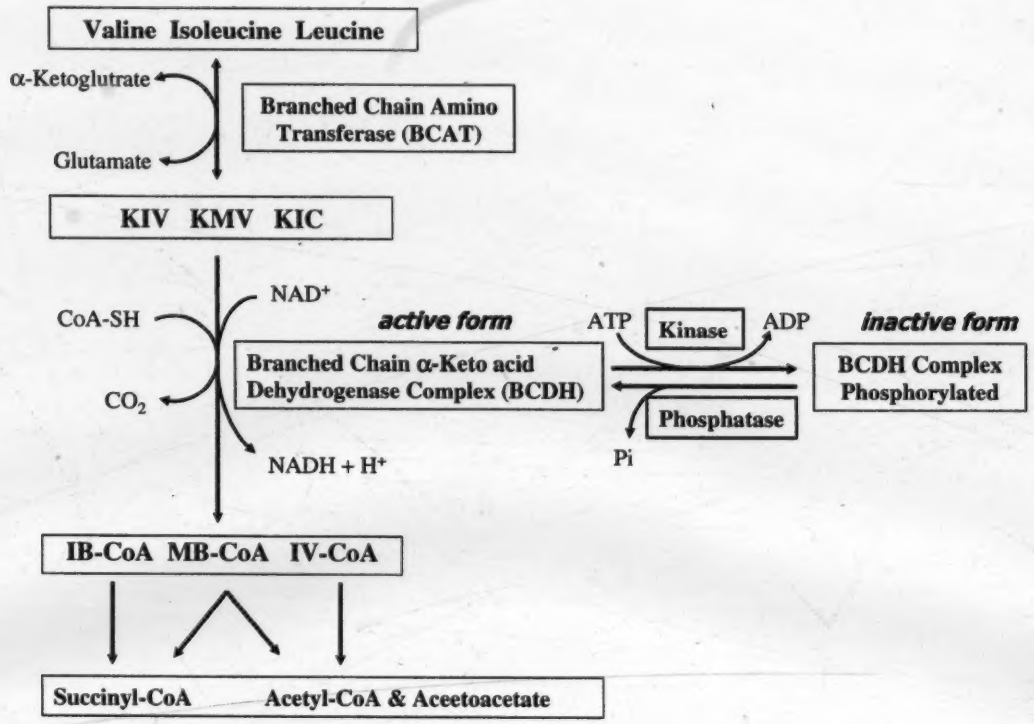

Figure 1.2 Regulation of branched-chain dehydrogenase (BCDH) activity by BCDH- kinase and BCDH-phosphatase

Degradation of BCKA, catalyzed by the BCDH complex is tightly regulated. The BCDH exists in active and inactive forms (Figure 1.2). The forms are interconverted by phosphorylation via a specific kinase (BCDH Kinase), and de-phosphorylation reactions, catalyzed by a phosphatase (BCDH Phosphatase). Phosphorylation leads to inactivation and dephosphorylation leads to activation (Suryawan et al, 1998). In rats, the BCDH present in the skeletal muscle is almost totally in the inactive form, but in the human skeletal muscle, a higher proportion of the dehydrogenase is in the active form (**Table 1.3**). Most of the other tissues like liver, heart, kidney have the enzyme mostly in the active form (Skeie et al, 1990). The BCDH activity also varies between species and among organs (Table 1.3). Activity in rat liver is significantly higher than all other organs, but in humans the activity is highest in kidneys.

BCDH complex activity state is regulated by dietary protein, calorie intake, individual BCAA concentrations, exercise, insulin and female sex hormones (Shimomura et al, 2001). Regulation is principally shown to occur via increased or decreased BCDH kinase activity. Under conditions of low protein diets and low calorie intake, BCDH kinase activity is upregulated, and thereby the BCDH complex is predominantly in the inactive state (Popov et al, 1995). Inactivation of the BCDH complex provides a mechanism for conservation of BCAA for protein synthesis under these conditions of decreased protein and energy intake. Exercise, especially endurance exercise, increases energy expenditure and results in the stimulation of BCAA catabolism. BCDH activity is stimulated in both human and rat skeletal muscle due to

Table1.3 Distribution of branched-chain dehydrogenase (BCDH) activity in rat, human and monkey tissues

Tissue	Rat[1]	% active	Human[2]	% active	Monkey[3]	% active
	mU/g wet wt	%	mU/g wet wt	%	mU/g wet wt	%
Heart	483.2 ± 19.3	16	8.2 ± 0.8	40	NA	
Muscle	50.8 ± 2.9	7	4.9 ± 0.9	26	9.7 ± 0.9	59
Brain	39.5 ± 2.4	77	10.9 ± 0.8	59	24.9 ± 2.3	22
Liver	989.2 ± 81.5	88	14.8 ± 1.3	28	78.5 ± 8.3	76
Kidney	578.1 ± 24.9	76	110.9 ± 8.6	14	92.7 ± 8.5	86
Pancreas	156.8 ± 9.8	28	NA	-	13.0 ± 1.2	59
Stomach	94.5 ± 6.5	71	4.8	38	11.4 ± 1.2	28
Small Intestine	2.6 ± 0.2	69	1.6 ± 0.2	44	1.9 ± 0.2	53
Colon	14.9 ± 1.3	53	5.7 ± 0.3	40	NA	-
Adipose	35.6 ± 2.6	57	2.7 ± 0.3	41	3.6 ± 0.4	53

Note: Values are mean ± SEM; NA, not available

[1] Rats weighing 250-300g

[2] Patients undergoing surgical procedures, 2 mo to 68 yrs

[3] African green monkeys aged 4-5yrs, weighing ~4kg

Source: Suryawan et al (1998)

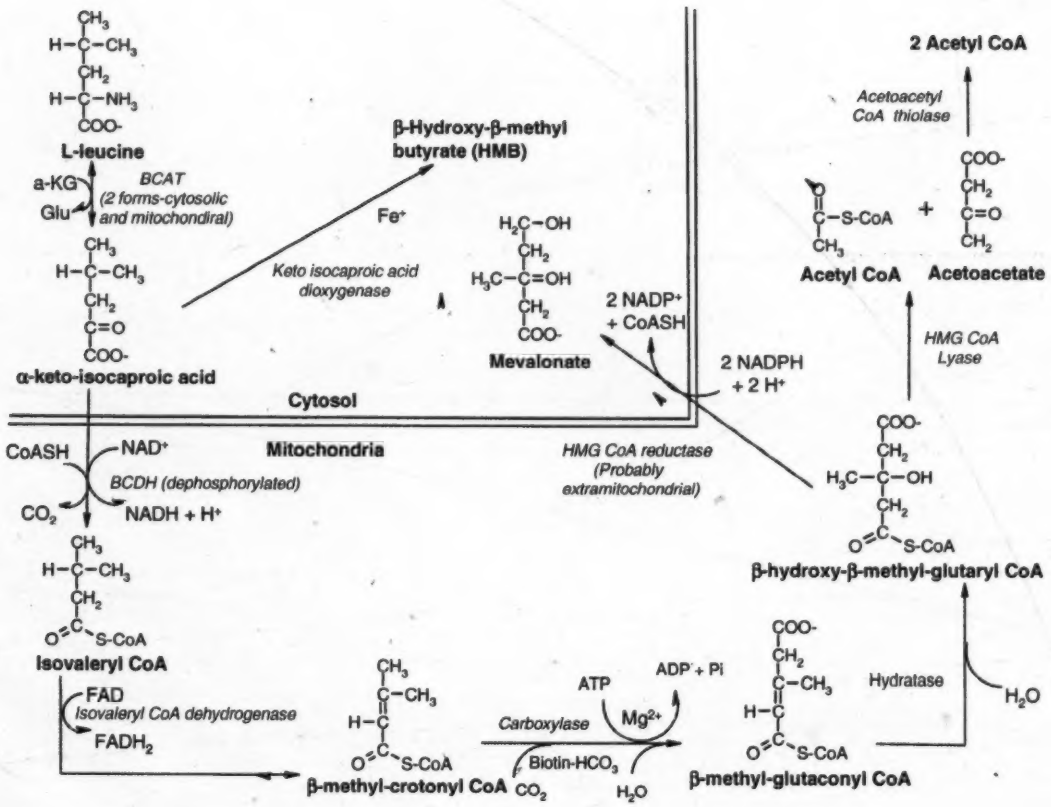

Figure 1.3 Leucine catabolism in mammals

exercise (Shimomura et al, 2001). Leucine and isoleucine concentrations have been shown to stimulate the BCDH complex (Aftring et al, 1986). KIC is able to stimulate BCDH activity by acting as a potent inhibitor of the BCDH kinase, but KIV and KMV have no effect (Harris et al, 1994). Thus, it appears that leucine and KIC play a significant role in the regulation of the BCDH complex.

1.2.1.3 Ketoisocaproic (KIC) dioxygenase

In addition to the two key enzymes in BCAA catabolism, a cytosolic KIC preferring oxygenase has been reported in rat liver and kidney (Harper et al, 1984) and human liver (Sabourin & Bieber, 1983). KIC dioxygenase has thus far only been shown to be present in the cytosol and catalyzes the formation of β-hydroxy-β-methylbutyrate (HMB) from KIC (**Figure 1.3**). KIV and KMV do not serve as substrates for the dioxygenase, but α-keto-γ-methiolbutyrate (ketoacid of methionine) is actively decarboxylated in rat liver. Together with the leucine –methionine specific aminotransferase (described previously), KIC dioxygenase probably provides an alternative pathway for the catabolism of leucine. In rats this pathway accounts for 15% of KIC oxidation (Harper et al, 1984). In ~20kg pigs, van Koevering & Nissen (1992) observed that HMB oxidation due to leucine infusion was 2-10% of the leucine oxidation in the post-absorptive state. Although HMB has been suggested to be involved in cholesterol synthesis (van Koevering & Nissen, 1992) and also act as an inhibitor of proteolysis (Xu et al, 2000), their exact fate in-vivo remains to be elucidated. In general, KIC dioxygenase pathway and its influence on leucine metabolism needs further examination.

1.2.2 Interorgan Relationships in BCAA and BCKA Metabolism

BCAA are transported into cells via a specific carrier termed the Large Neutral Amino Acid (LNAA) carrier (L system) (Skeie et al, 1990). Thus, the BCAA compete for uptake with the other LNAA, aromatic amino acids, phenylalanine, tyrosine and tryptophan, and also methionine. Upon being taken up into the intracellular pool of a tissue, BCAA are predominantly used for protein synthesis or undergo transamination to yield BCKA and glutamate.

Based on the compartmentation of BCAA catabolizing enzymes in rats various hypothesis for BCAA catabolism have been put forth. BCAT is present both in the cytosol and in the mitochondria, whereas BCDH occurs only in the mitochondria. Thus, part of the BCKA formed in the transamination reaction may pass from the cytosol into the blood, rather than into the mitochondria. From here it could be transported to other organs. The nitrogen released during catabolism of BCKA in various tissues must be transported to the liver for conversion to urea. Hence, interorgan co-operativity is hypothesized in the disposal of both the nitrogen and the carbon of the BCAA (Harper et al, 1984).

Based on the organ differences and activity state of BCAT and BCDH in the rat it has also been hypothesized that during a protein meal, BCAA escape intact from the splanchnic area to be transaminated in the muscle. In rats, skeletal muscle has relatively higher BCAT activity and almost no BCDH activity. Thus, the ketoacids are transported to the liver for decarboxylation, because liver has significantly high BCDH activity compared to most other organs. BCAA transamination and oxidation appear to

occur at different sites with substantial shuttling of BCAA metabolites between skeletal muscle and liver in the rat (DeSantiago et al, 1998). But comparing BCAA enzyme activities in Table 1.1 and Table 1.2 suggests that a different pattern for BCAA catabolism may exist among species. In humans, both liver and muscle possess BCAT and BCDH enzymes in relatively equal proportions. Therefore interorgan catabolism of BCAA and its metabolites may not be as significant in humans as in rats. Insufficient information exists for pigs to determine which species they are more similar to.

With respect to BCAA escaping intact from the splanchnic area, conflicting evidence exists in species other than rats. In adult humans, arterio-venous tracer difference studies indicated that 26% of the orally administered leucine was taken up by the splanchnic organs at first pass, whereas first pass deamination of dietary leucine and release as KIC was minimal (Biolo & Tessari, 1997). But Matthews et al (1993) in adult humans estimated that 40% of the nasogastric leucine tracer was sequestered on first pass and converted to KIC and released. In this study, the researchers estimated that about 50% of the first pass leucine uptake was incorporated into newly synthesized proteins. Furthermore, Matthews et al (1999) also examined KIC delivery via nasogastric route and estimated that 84% of the KIC was extracted in first-pass and transaminated to leucine and released. Although the estimates of BCAA metabolism in the splanchnic region vary between studies, it is clear that BCAA do not pass intact from the splanchnic organs in all species. The impact of splanchnic organs (especially small intestine and liver) on BCAA requirements is not known.

1.2.3 BCAA Antagonism

In the first reported study of BCAA antagonism, high levels of leucine (3%), in a protein deficient diet (9% casein) caused marked growth depression in rats (Harper et al, 1984). The addition of isoleucine partially reversed the growth depression. In later experiments it was noticed that at an adequate intake of protein (18% casein), 3% leucine did not cause growth depression. High levels of isoleucine and valine were also experimented on to determine whether an antagonistic effect was noticed. A similar effect was seen, when the basal diet was modified to make leucine the growth limiting amino acid (Torres, et al, 1989). Thus, mutual antagonism does occur between the three BCAA, with leucine induced BCAA antagonism being the most commonly observed phenomena in animals and humans.

1.2.3.1 Leucine Induced Antagonism

Excessive intakes of leucine, in a protein inadequate diet causes not only a marked depression in growth, but also a drop in the plasma and tissue pools of isoleucine and valine, along with their corresponding ketoacids, keto-methylvalerate (KMV) and keto- isovalerate (KIV) respectively. The depression of isoleucine and valine pools is very acute, occurring 10 – 30 min, after an intragastric load of leucine (Block, 1989). Supplementation of isoleucine and valine to the diet will only partially reverse the growth depressing effects and further additions of phenylalanine, tryptophan and threonine are required to normalize growth. This indicates that consumption of excess leucine along with a low protein diet might increase the requirements for amino acids other than isoleucine and valine (Harper et al, 1984). A depression in food intake is also noticed in

these animals, and experiments have suggested that almost 70% of the growth depression is due to a decrease in food intake. But the depression in food intake is not acute after a leucine load and is normally seen between 1 and 3h of leucine administration.

Thus an order dependent sequence can be noticed in leucine induced antagonism.

1) Elevation of total body free pools of leucine and it's ketoacid - KIC
2) Depletion of total body free pools of isoleucine and KMV, valine and KIV,
3) And depression of food intake and growth (Harper et al, 1984).

The antagonistic effect of leucine is unique in that the effects are not observed at a high protein intake, also, increased concentrations of isoleucine and valine do not increase plasma leucine concentrations. The route of supply of branched chains too did not alter the antagonistic effect of leucine, because intravenous infusions of individual BCAA and their effects on antagonism were similar to the oral route (Snyderman et al, 1968). Leucine induced antagonism has been shown to occur in a number of species, including, rats, chicks, pigs, turkey poults and humans. The sensitivity differs between species, as indicated by Hargrove et al (1984). These researchers did not find a similar antagonistic effect of increased leucine in kittens and it was attributed to a constant and relatively higher rate of nitrogen metabolism in kittens allowing them to have greater tolerance for an excess of leucine.

Three possible mechanisms have been proposed for the plasma amino acid changes occurring during leucine induced BCAA antagonism. First, increased protein turnover could be taking place (increased protein synthesis, decreased protein degradation, or both). Second, transport competition among all amino acids using the

same carrier as leucine, (the L-system of amino acid transport used by all the large neutral amino acids – LNAA) and third, increased BCAA oxidation might cause the decrease in isoleucine and valine (Lal & Chugh, 1995).

The issue of protein synthesis being modulated has been validated by studies indicating that leucine is a potential stimulator of protein synthesis in-vitro and in-vivo (Kimbal & Jefferson, 2002). Leucine has been shown to regulate protein synthesis at the mRNA translation level (Lynch, 2001). Insulin regulates mRNA translation via the mTOR (mammalian Target of Rapamycin) pathway. Recently, leucine has been shown to stimulate translation initiation in a similar mTOR sensitive as well as, an mTOR insensitive pathway (Kimball & Jefferson, 2002). Increased net protein synthesis at the present cannot be assumed to be the main mechanism by which the depletion of other free amino acid pools occurs because all the amino acids affected by leucine load have varied pool sizes and an accurate measurement of absolute changes in pool sizes must be compared before further conclusions can be made (Block, 1989). Additional controlled studies are needed to examine BCAA antagonism and their impact on protein synthesis.

Transport competition exists between all the amino acids using the same carrier, in this case, the L-system, to transport the amino acids into and out of cells. But this is an improbable explanation because transport competition is not substantial among the LNAA in muscle, where depletion of isoleucine and valine pools is severe. Also, if there is inhibition of uptake in the muscle for isoleucine and valine, caused by leucine, then it would elevate rather than depress the plasma pools of isoleucine and valine (Torres et al, 1995).

The third possibility, that increased overall BCAA oxidation leads to depressed levels, seems probable. Harper and associates (1984) suggested that the increased leucine supplied, transaminates to form KIC, and increased KIC levels stimulate transamination of valine and isoleucine. This increases the branched-chain dehydrogenase activity, thereby cell channeling of the ketoacids of valine and isoleucine into their catabolic pathways takes place (Torres et al, 1993).

The exact mechanism of antagonism is yet to be defined in the case of leucine induced BCAA antagonism. Due to the complex and unique metabolic compartmentation of BCAA and it's metabolites, BCAA antagonism has challenged researchers for more than five decades. From a clinical point of view, this concept is very important, because, BCAA enriched formulas are widely used in the clinical setting to manage patients under trauma, sepsis, chronic renal failure, liver cirrhosis and burn victims (Skeie et al, 1990). Clinicians should be aware of the antagonistic effects caused by leucine under a chronic excess infusion, especially if protein intake is not adequate. Determination of BCAA requirements and optimum ratio among BCAA in healthy populations being fed via different routes of infusion is a good starting point to examine the physiological reasons behind BCAA antagonism.

1.2.4. BCAA Requirements in Piglets and Human Infants

Snyderman and colleagues in the late 1950's and early 1960's conducted the only direct examinations of BCAA requirements in infants (**Table1.4**). In all these studies the infants were fed a synthetic mixed amino acid diet in the same proportion of

Table 1.4 Branched-chain amino acid (BCAA) requirements in human infants and piglets

Amino Acid	Snyderman et al (1959, 1961, 1964)[1]	FAO/WHO/UNU (1985)[3]	DRI Committee (2002)[4]	NRC Swine (1998)[5]
	g/100 g protein			
Isoleucine	3.6 – 5.7	3.2	4.0	2.5
Leucine	3.5 – 10.4	7.3	7.1	4.6
Valine	3.9 – 4.8	4.2	4.0	3.0
Ratio (ile/leu/val)	1:2.9:0.8[2]	1:2.3:1.3	1:1.8:1	1:1.8:1.2
Total BCAA	11 – 20.9	14.7	15.1	10.8

[1] Data from 6 mo to 1 yr infants

[2] Ratio calculated from upper recommended intake for BCAA

[3] Recommendations for 0-6 mo infants based on N balance studies

[4] Recommendations for 0-6 mo infants based on factorial calculations

[5] Recommendations for 3-5kg piglets extrapolated from 10kg piglets

amino acids found in breast milk. The intake of the individual BCAA was completely withdrawn from the supply and then re-introduced in a stepwise fashion to determine the minimal intake of individual BCAA, which would influence normal weight gain and nitrogen retention. These studies have proven invaluable because they are directly derived. However, the disadvantages and shortcomings of the nitrogen balance technique they used are well known and reviewed extensively (Pencharz & Ball, 2003, Zello et al, 1995). Furthermore, as stated by Snyderman et al (1961), "..we have encountered more variation in the requirement of infants for leucine than in any other amino acid studied thus far", the possibility of BCAA antagonism influencing these results are high. In a rapidly growing infant individual deficiency or excess of any one BCAA in the above mentioned studies would have caused the variation in requirement estimates observed by the authors. The small sample size and variability in age of the subjects in these studies may also have contributed to the less sensitive requirement estimate.

The FAO/WHO (1985) and DRI committee (2001) requirement estimates for infants are derived by a factorial approach from infants fed human milk as the principal source of nutrients (Table 4). The factorial approach depends upon the accuracy of the assumptions for dietary intake and concentrations of milk, growth rate, body composition etc. For example, at the mean concentration of isoleucine, 678mg/L (in human milk), and an average intake of human milk for infants 0-6 months, the requirement for isoleucine is derived by, 678 mg/L x 0.78L/day which equals 529mg/day or 0.88g/kg/day (Table 1.4).

The NRC (1998) recommendations for 1 to 5kg neonatal piglets (Table 1.4), were calculated from data obtained using older pigs (10kg), and are also based on

growth and nitrogen balance studies. Thus, direct examination of BCAA requirements in neonatal piglets is necessary.

1.3 BCAA in Clinical Nutrition Support

BCAA supplemented infusions, both parenterally and enterally in clinical practice has been on the increase, as BCAA have been proposed to be beneficial to patients suffering from a wide range of catabolic disease conditions (Skeie et al, 1990). The current clinical evidence is conflicting and will be discussed briefly. Most of the implications of BCAA supplementation are in older children and adults. But information could be gained about the importance of route of infusion of BCAA, as well as BCAA metabolism during catabolic situations.

1.3.1 BCAA in Liver Failure

The role of BCAA in liver failure has been reviewed recently (Mager 2003), therefore only a brief review is included here. BCAA supplemented solutions were originally advocated and used therapeutically for patients with hepatic failure and encephalopathy (Skeie et al, 1990). There are several reasons why the provision of BCAA may potentially be useful in liver disease: patients with liver disease are catabolic, they have smaller glycogen stores, they are insulin resistant, and they have a decreased rate of hepatic ketogenesis from fatty acids (Marchesini et al, 2003). Plasma BCAA levels are decreased and aromatic amino acid (AAA), phenylalanine and tyrosine concentrations are increased in patients with liver cirrhosis (Blonde-Cynober et al, 1999). Supplementation of BCAA normalizes the AAA concentrations, but BCAA

concentrations remain depressed. BCAA supplementation also significantly reduces signs of hepatic failure such as hypoalbuminemia and hyperammonemia (Kajiwara et al, 1998). Thus BCAA might be useful as an energy source, and have an increased effect on protein turnover, which may decrease muscle breakdown and promote protein synthesis (Freund et al, 1978). These effects might ameliorate the whole body catabolic state and improve regeneration of the liver in liver diseases (Marchesini et al, 1982). Recently, in children Mager et al (2003) determined that the impact of cholestatic liver disease increased BCAA requirement by 42% when compared with healthy children. Furthermore, Mager et al (2003) also showed that the increased requirement for BCAA was due in part to increased leucine oxidation in children. During the comparison of BCAA requirements in children the dietary BCAA intake was in the same proportions present in egg protein.

1.3.2 BCAA in Chronic Renal Failure

BCAA in renal failure has been reviewed recently by Mak (1998). Chronic renal failure, like hepatic encephalopathy, is characterized by decreased concentrations of plasma BCAA and BCKA in children (Broyer et al, 1980 and Canepa et al, 1992). The mechanism is yet to be fully understood, but it may reflect increased peripheral uptake, perhaps mediated by insulin (Mak 1998). When balanced supplements of essential amino acids or supplements containing increased proportions of BCAA were given to children plasma BCAA did not increase significantly and there was considerable clinical improvement (Jones et al, 1983). Low protein diets supplemented with BCAA or BCKA seem to counteract secondary hyper-parathyroidism and retard the progression of chronic renal failure. The lowering of urea production rate by the BCAA may be of significant

benefit in renal failure (Skeie et al,1990). The benefit of BCKA supplementation is not observed in a normal protein intake diet. Comparison of studies between BCAA and BCKA supplementation in children has led to controversial data, and Laouari & Parvy (1995) suggest that this could be partly due to differing intakes of BCAA, BCKA and protein intake levels. These researchers suggested that current evidence does not favor supplementation of BCAA or BCKA in chronic renal failure before determining the BCAA requirements in such populations.

1.3.3 BCAA in Metabolic Stress States

Several animal and human studies have indicated beneficial effects of the use of BCAA enriched solution in trauma, injury and sepsis (Skeie et al, 1990). The properties of BCAA as energy substrates for gluconeogenesis, and modulators of muscle protein metabolism make the use of BCAA-enriched solutions theoretically appropriate for the management of the metabolic alterations that occur in sepsis (Lorenzo et al, 1997). The use of BCAA to decrease muscle catabolism in injured and septic patients has been primarily based on the in vitro observation that BCAA may inhibit muscle protein degradation and stimulate synthesis. In critically ill and septic patients, BCAA-enriched solutions have been found to increase the rate of hepatic protein synthesis. These proteins may be important in host defense mechanisms against infections as suggested by Kikuchi et al (1987). During severe sepsis, changes in the plasma concentrations of aromatic amino acids result in an increase in the aromatic/BCAA ratio. If the change in this ratio is severe, it may result in encephalopathy, which is similar to hepatic encephalopathy. The

BCAA-enriched solutions have been shown to correct the ratio of AAA/BCAA in sepsis (Jeppsson et al, 1981).

1.3.4 Other Effects of BCAA under Clinical Conditions

BCAA enriched infusions have also been shown to increase respiratory drive, decrease sleep apnea, stimulate food intake and stimulate gastric emptying. These interesting findings do not appear to have translated into useful therapies (Wagenmakers and Soeters, 1995). Administration of BCAA had been shown to attenuate the stimulation of respiration by amino acids, possibly mediated by competing with tryptophan for uptake across the blood brain barrier (Takala et al, 1988). This competition may decrease brain uptake of tryptophan, which serves as a precursor for serotonin, and thereby decreasing the brain serotonin concentration, a central respiratory depressant (Kirvela et al, 1990). Serotonin has also been implicated as a neurotransmitter in the control of food intake (Fernstorm, 1985). Parenteral nutrition has been shown to have an appetite suppressive effect. Gil et al (1990), hypothesized that BCAA-enriched solutions might also attenuate the appetite suppressive effect of TPN via the same serotonin mechanism.

In a further study by Myttenaere et al (1994), they hypothesized that the control of food intake is partly mediated through changes in gastric emptying (GE). The rate of GE determines the duration for which absorption of a meal sustains metabolic satiety and influences gastric distension, which can be a source of innate satiation. Therefore inhibition of GE could be a mechanism by which oral food intake is reduced in patients receiving parenteral nutrition. The rate of GE of a liquid test meal was tested on healthy human subjects, (Myttenaere et al, 1994) and the subjects receiving BCAA

enriched parenteral nutrition had an increased rate of GE, as compared with the subjects receiving standard parenteral nutrition. The use of a BCAA enriched formula that stimulates GE relative to the infusion of standard parenteral nutrition may be useful for patients with nausea or who are at risk of reflux and aspiration of gastric contents. Furthermore, BCAA could also be used to stimulate oral intake in patients during short term post operative TPN.

1.3.5 BCAA Supplemented Parenteral Nutrition and Gut Atrophy

Under conditions of parenteral nutrition, the gut undergoes a number of morphological changes when deprived of its normal luminal nutrients (Platell et al, 1991). There is a decrease in gut weight, mucosal thickness, mucosal protein and DNA content, and crypt cell production rates (McCauley et al, 1996). Intestinal motility is also reduced and there is a decrease in the synthesis and secretion of gastric, pancreatic and biliary secretions (Rombeau and Rolandelli, 1987).

Endogenous glutamine synthesis and release may be stimulated by the infusion of solutions enriched with BCAA and has been implemented in some clinical situations. Platell et al (1991) studied the effect of infusion of glutamine, BCAA and both glutamine plus BCAA on mucosal atrophy in the jejunum of rats. They observed that the BCAA group had a greater N retention, greater crypt cell production rate, mucosal weight, mucosal protein and higher alanine concentrations, than the group given standard PN. The glutamine fed group had increased jejunal weights, mucosal DNA concentration and higher crypt cell production rates. These results indicate that the infusion of glutamine plus BCAA enriched parenteral nutrition has a more beneficial effect on the

morphology of the jejunum than that of the standard PN. Infusions of BCAA have been shown to increase plasma alanine and glutamine concentration (McCauley et al, 1996). In the study by Platell et al (1991) the increase in plasma glutamine concentration failed to achieve statistical significance. However, a main byproduct of glutamine metabolism by enterocytes (alanine) was found to have a significantly increased venous plasma concentration.

In a further study by Platell and colleagues (1993), they studied the effect of infusion of BCAA and glutamine in varied doses in rats, ranging from 0.5% to 2.5% glutamine, and 1.0% to 2.0% BCAA. The normal levels of BCAA in standard parenteral nutrition are 0.27%. The glutamine enriched group showed an increase in plasma glutamine and alanine in a dose dependent manner. But the BCAA enriched group showed, in a dose dependent manner, an increase in plasma alanine concentration. There was a much more obvious dose-response relationship between the concentration of glutamine and BCAA and the attenuation of gut atrophy; as indicated by an increased gut weight, mucosal weight and mucosal protein content. The glutamine plus BCAA enriched solutions were more efficacious than either nutrient alone at increasing gut, mucosal weight and mucosal protein (Platell et al, 1993).

BCAA supplementation studies during liver disease, renal disease and the various catabolic states have mostly been controversial. The reason could be due to population heterogeneity, and different ways of assessing clinical outcome variables. But more importantly a wide range in BCAA concentrations and ratios among BCAA were

tested by different routes of feeding making recommendation for BCAA supplementation difficult.

The quantity of literature on BCAA is very large. However, there are surprisingly few experiments that have used an objective and direct measure of BCAA requirement, and none at all were found for neonates. Thus, the first goal should be to directly determine the BCAA requirements for protein synthesis in neonates during different routes of infusion. This would establish the minimum BCAA intake and enable subsequent supplementation studies to be conducted using distinct outcome variables from a clinical perspective. Thus, knowledge of the appropriate concentrations of BCAA to be supplemented would allow us to manipulate metabolism and the protein synthetic machinery in a wide variety of conditions.

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2.0 RATIONALE AND OBJECTIVES

2.1 Rationale

Premature infants often require parenteral feeding, due to their inability to tolerate oral or enteral feeding. Currently available parenteral formulas have an inappropriate balance of amino acids to maximize nitrogen efficiency. Branched-chain amino acid [BCAA; isoleucine, leucine and valine]constitute a significant proportion of the total indispensable amino acid intake in mammals. However, BCAA requirements in neonates during parenteral and enteral routes of feeding are unknown. Recent research has consistently shown that requirements for amino acids are significantly altered by the small intestine leading to different requirement estimates during parenteral and enteral routes of feeding. Furthermore, BCAA have been shown to display antagonistic effects when one or more of the individual BCAA are in excess or deficiency. Thus, it is crucial to examine the BCAA requirements in relation to each other, as well as the appropriate ratio among BCAA. Branched-chain amino acids are different from other essential amino acids, in that their catabolism has traditionally been thought to begin by the reversible transamination in skeletal muscle and the keto-acids formed are transported to liver for decarboxylation and further catabolism. Amino acid catabolism by the small intestine, especially with respect to the BCAA, has been largely overlooked. Based upon these issues, a series of hypotheses were developed to guide this thesis research.

2.2 Specific Hypotheses

1. The total BCAA requirement will be different for parenterally and enterally fed neonatal piglets.

2. The ratio among the BCAA will be different and specific for parenterally and enterally fed neonatal piglets.

3. Small intestinal epithelial cells (enterocytes) have the capability to catabolize BCAA to CO_2

4. BCAA catabolism in neonatal piglet tissues will be influenced by developmental changes taking place during early postnatal stages

2.3 Objectives

The first objective of this research will be to determine the total BCAA requirements, provided in a fixed ratio, during parenteral and enteral nutrition. If differences in total BCAA requirements exist due to route of feeding this will indicate that small intestinal metabolism of BCAA occurs. The second objective will be to determine whether the ratio among the BCAA is the same for both parenteral and enteral routes of feeding. Methods will then be developed to examine BCAA catabolic enzyme activity and conduct in-vitro oxidation studies in isolated enterocytes. The final objective will be to determine BCAA catabolic enzyme activity and relative oxidation rates in neonatal piglet tissues during early stages of postnatal growth.

3.0 THE BRANCHED-CHAIN AMINO ACID REQUIREMENT OF PARENTERALLY FED NEONATAL PIGLETS IS LESS THAN THE ENTERAL REQUIREMENT

3.1 Introduction

Over the past 3 decades, total parenteral nutrition (TPN) has become an important adjunctive therapy in a variety of disease states. TPN formulations are extremely complex admixtures containing amino acids, dextrose, lipids, water, electrolytes, minerals, trace elements and vitamins (National advisory groups on standards and practice guidelines for parenteral nutrition, 1998). The primary objective of TPN lies in the maintenance or improvement of the nutritional and metabolic status of patients, who for a significant period of time cannot be adequately nourished by oral or enteral feeding (Shills, 1994).

Low birth weight (LBW) infants constitute a majority of the patients who often have an inability to tolerate enteral feedings, due to a variety of factors including, short bowel syndrome, gastrointestinal surgery, chronic severe diarrhea, immature bowel function and respiratory diseases (Ball et al, 1996 and Heird & Gomez, 1993). Thus, TPN regimens are continuously being refined to meet the infant's need for growth and development without placing too much stress on their immature biochemical and physiological systems. An "optimal" profile for neonatal TPN that provides amino acids in a combination maximizing protein accretion and growth, and minimizing amino acid degradation has not been established for the neonate (Brunton et al, 2000 and Wykes et al, 1993).

Experiments designed to determine amino acid requirements or kinetics are currently being planned in infants, but prolonged dietary treatments which are deficient in indispensable and conditionally indispensable amino acids could endanger the infant (Ball et al, 1996). The piglet model developed by our group (Wykes et al, 1993) to study amino acid kinetics and requirements during TPN is more practical, allows serial blood measurements and the requirements for threonine (Bertolo et al, 1998), lysine (House et al, 1998), phenylalanine (House et al, 1997a), tyrosine (House et al, 1997b), methionine (Shoveller et al, 2003) and tryptophan (Cvitkovic et al, 2004) have so far been determined using the Indicator Amino Acid Oxidation (IAAO) method.

The branched chain amino acids (BCAA), consisting of isoleucine, leucine and valine, have been implicated to be predominantly metabolized by extra hepatic tissues (Harper et al, 1984). Hence, BCAA research interest has largely focussed on muscle metabolism, although there have been reports of splanchnic uptake of BCAA in humans (Gelfand et al, 1986, Cortiella et al, 1988 and Hoerr et al, 1991). Recently in both pigs (Stoll et al, 1998 and van der Schoor et al, 2001) and dogs (Yu et al, 1995) there has been increased evidence for splanchnic metabolism of leucine. The BCAA also exhibit antagonism, where excessive intakes of leucine in young growing rats on a protein restricted diet antagonize the utilization of the other two BCAA (Harper, 1956). Thus maintaining an appropriate ratio between the BCAA is very important.

TPN feeding bypasses the gut and thus the parenteral requirement of many amino acids varies from the enteral requirement (Brunton et al, 2000). We have shown in previous experiments by the IAAO technique that the parenteral amino acid requirements are lower than enteral requirements in the case of threonine, lysine and phenylalanine.

(Bertolo et al, 1998, House et al, 1998 and House et al, 1997a). In the present study our hypothesis was that the parenteral and enteral requirements for total BCAA in neonatal piglets would be different. A lower parenteral requirement for total BCAA would indicate uptake of BCAA by the splanchnic tissues during first pass intestinal metabolism.

3.2 Materials and Methods

3.2.1 ***Animals and study protocol:*** The Animal Care Committee of the University of Alberta approved all procedures used in this experiment. A total of 32 male Yorkshire piglets, weighing approximately 1.5 kg and, 1 to 2 d old were transferred to the Metabolic Research Facility at the University of Alberta. The piglets were weighed, anaesthetized for the surgical implantation of catheters (Ed-Art, Don Mills, Canada). During surgery anesthesia was maintained on 0.8% Halothane. Venous catheters were placed following modified procedures of Wykes et al (1993) and Rombeau et al (1984) for gastric catheters. The surgical procedures have been recently described by Bertolo et al. (1998). After surgery, the animals were fitted with adjustable cotton jackets, which prevent tangling and occluding of the catheters. The laboratory conditions and piglet housing were described previously (Wykes et al, 1993).

3.2.2 ***Diet regimen:*** The composition of the elemental and complete diet was based on the initial formulation by Wykes et al (1993), with modifications (Chapter 8, Table 8.1.1 and 8.1.2) . Diet was infused (continuous, 24h) using infusion pumps via a tether-swivel system (Alice King Chatham Medical Arts, Los Angeles, CA) intravenously (Experiment 1 - parenteral BCAA requirement) or intragastrically

(Experiment 2 – enteral BCAA requirement). At full infusion rate (272 ml/(kg · d), the complete diet provided 1.1 MJ available energy per /(kg · d) and 14.6, 27.4, 9.4 g of amino acids, glucose and fat per kg body weight per day, respectively. The base amino acid profile of the diet was as described previously (Bertolo et al, 1998) and the addition of the BCAA to make the test diets is described below. Following surgery all piglets were administered the complete diet intravenously, at 50% of the full rate for approximately 6h and then at 75% overnight, counting the surgery day as d 0. On d 1, in Experiment 1, the diet was infused at full rate intravenously. In Experiment 2, the piglets were switched to 50 % of full rate intragastric feeding on d 1 and the intragastric infusion rate was increased to full rate by d 2 (and the intravenous feeding was discontinued) and maintained until d 5.

3.2.3 ***Test diets:*** On d 5, the piglets were randomly assigned to receive one of the 9 test diets (Experiment 1) or 7 test diets (Experiment 2), containing graded levels of BCAA. The test levels of total BCAA ranged from deficient to excess based on the National Research Council (NRC), Nutrient Requirements for Swine (1998) for piglets weighing between 1 and 5 kg. The test levels were [Experiment 1: 0.2, 0.5, 0.8, 1.1, 1.4, 2.0, 2.6, 3.2 or 3.8 g/(kg · d); Experiment 2: 0.2, 0.8, 1.4, 2.0, 2.6, 3.2 or 3.8 g/(kg · d)]. The ratio between the three BCAA were kept constant in all test diets, at 1:1.8:1.2 of isoleucine/leucine/valine based on NRC (1998). At the completion of d 6 oxidation, the piglets returned to complete diet for 24 h. On d 7, 2100h, the piglets were randomly assigned to another test diet level. This method has been used in order to decrease the minimum number of piglets required per study, by conducting two oxidations per animal (d 6 and d 8). We have verified that this procedure does not alter

oxidation rate on d 8 when corrected for background radioactivity (Brunton et al., unpublished data).

3.2.4 *Tracer infusion, sample collection and analytical procedures:*

Details of the infusion protocol, $^{14}CO_2$ and blood collection have been described previously (House et al, 1997a). Briefly, on d 6 and 8, the piglets were transferred to plexiglass boxes, approximately 16 – 18.00 h after start of test diet infusion. A 30 min period was allowed for the piglets to acclimatize and the CO_2 to equilibrate in the chamber, following which phenylalanine flux and oxidation were determined by a primed [186 kBq (5 μCi) / kg], constant infusion [130 kBq (3.5 μCi) / (kg · h)] of a tracer solution containing 92.5 kBq (2.5 μCi) / mL of L – [1-^{14}C]phenylalanine (American Radiolabeled Chemicals, Inc. St. Louis, MO) . Air was drawn from the boxes by a pump and the total amount of $^{14}CO_2$ expired was trapped in a series of gas washing bottles containing CO_2 absorber (ethanolamine and ethylene glycol monomethylether, 1:2, v/v). Blood samples (1.5 mL) were drawn at time 0 and every half h during the 4 h study. The blood samples were centrifuged, plasma collected and stored at - 80°C, until analysis of phenylalanine specific radioactivity (SRA) and amino acid concentrations. On d 8, a 5 h study was conducted; the first hour was utilized for collection of background enrichment, prior to the primed, constant infusion. Immediately upon completion of the oxidation study on d 8, the piglets were injected with a lethal dose (750mg) of sodium pentobarbitol through the venous sampling line.

The rate of expiration of $^{14}CO_2$ was determined by liquid scintillation counting of radioactivity in the CO_2 absorber. The plasma concentrations of amino acids and SRA of plasma phenylalanine were analyzed by reverse-phase high performance

liquid chromatography (HPLC); collection and liquid scintillation counting of radioactive fractions were described previously (House et al, 1997a). Phenylalanine intake, flux, balance, percentage of dose oxidized, oxidation, nonoxidative disposal and release from protein breakdown were calculated as described previously (House et al, 1997).

3.2.5 ***Statistical analyses:*** Each experiment designed was a fully randomized approach with the test diet levels as the main treatment effect. Differences among test diet intakes within each experiment were determined by one-way analysis of variance (ANOVA) using the PROC GLM procedure (SAS/STAT version 8.1, SAS institute, Cary, NC). Significant differences between test diet intakes, if the P values were <0.05 for the F-value of the ANOVA model, were assessed by using Tukey's multiple comparisons procedure. The mean requirement for the BCAA in parenterally and enterally fed piglets was determined by break point analysis using a combined two-phase linear regression cross over model, modified from Ball and Bayley (1984) and Seber (1977). Regression variables included, as independent variable, the level of amino acid intake and phenylalanine oxidation [percentage of dose] as the dependent variable. The upper limit of the 95 % confidence interval (CI) of the breakpoint was estimated for each parameter to determine a safe level of intake.

3.3 Results

All piglets remained healthy, active and interested in the environment through the entire course of both the experiments. The initial weights (1.64 kg, pooled SD = 0.16), weight at study (2.64 kg, pooled SD = 0.29) and average daily gain (150g and 153g in

experiment 1 and experiment 2 respectively), were not significantly different among diet treatments or between routes of feeding.

3.3.1 ***Parenteral BCAA requirement:*** Phenylalanine flux (232.7 μmol/(kg · h), pooled SE: 38.4) and intake (111.4 μmol/(kg · h), pooled SE: 4.2), were not significantly different (P>0.05) across diet treatments, as expected and required by the IAAO technique. The lack of difference in flux indicates that the change in oxidation reflects a partitioning between oxidation and protein synthesis. BCAA intake significantly influenced phenylalanine oxidation expressed as a percentage of the dose oxidized (**Figure 3.1**). As the total BCAA intake increased from 0.2 to 1.1 g/(kg · d), phenylalanine oxidation declined (P<0.05). Further increases in BCAA intake from 1.4 to 3.8 g/(kg · d), did not significantly affect phenylalanine oxidation (P>0.05, slope not different from zero).

Aspartate, serine and glycine were used to make the diets iso-nitrogenous and therefore their intake and plasma concentrations were higher in the BCAA deficient diets and decreased significantly with increases in BCAA intake (**Table 3.1**). The concentrations of glutamine, histidine, citrulline, threonine, arginine, tyrosine, tryptophan and lysine decreased significantly with increases in BCAA intake (Table 3.1). BCAA intake had a significant influence on plasma phenylalanine concentrations, which decreased from 262 to 114 μmol / L as BCAA intake increased from 0.2 to 1.4 g/(kg · d), respectively (P<0.05); no significant changes in phenylalanine concentrations were observed with further increases in BCAA intake. Plasma concentrations of isoleucine, leucine and valine were significantly affected by increases in BCAA intake (**Figure 3. 2** and Table 3.1).

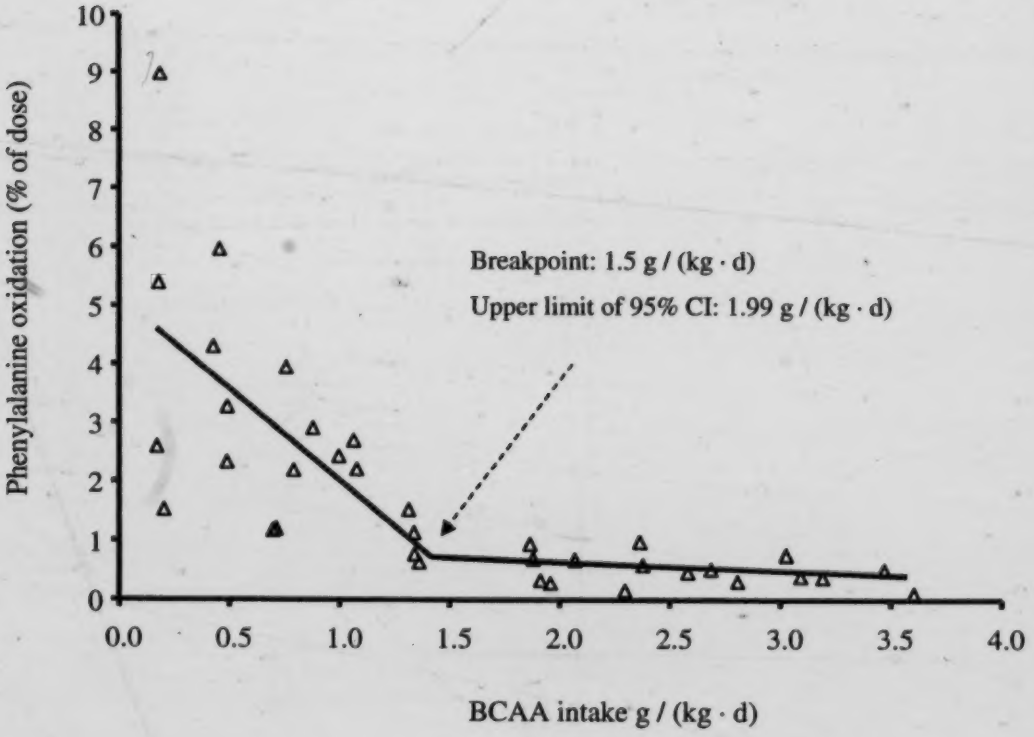

Figure 3.1 Oxidation of L-[1-^{14}C]phenylalanine as a percentage of dose in parenterally fed piglets receiving graded intakes of BCAA (n=36)

Table 3.1 Plasma amino acid concentrations of parenterally fed piglets receiving graded intakes of BCAA[1]

Amino acid (μ mol / L)	BCAA intake g / (kg · d) 0.2	0.5	0.8	1.1	1.4	2.0	2.6	3.2	3.8	SE	ANOVA p value
Histidine[2]	195^{a}	186^{a}	118^{a}	140^{a}	125^{a}	46^{b}	55^{b}	40^{b}	46^{b}	11	0.0009
Isoleucine[2]	33^{a}	48^{a}	70^{a}	95^{ab}	93^{ab}	107^{ab}	156^{ab}	198^{c}	232^{c}	23	0.0001
Leucine[2]	56^{a}	50^{a}	49^{a}	82^{ab}	87^{ab}	93^{ab}	176^{ab}	227^{c}	290^{c}	13	0.0001
Lysine[2]	1180^{a}	1251^{a}	1133^{a}	1088^{a}	1026^{a}	548^{b}	513^{b}	587^{b}	535^{b}	135	0.0045
Methionine	33	30	24	38	27	25	25	25	20	4	NS
Phenylalanine[2]	262^{a}	227^{a}	182^{a}	190^{a}	114^{ab}	67^{b}	77^{b}	59^{b}	59^{b}	9	0.0029
Threonine	849	882	856	684	898	559	616	564	515	121	NS
Tryptophan[2]	65^{a}	46^{a}	35^{ab}	40^{ab}	25^{b}	18^{b}	26^{b}	24^{b}	27^{b}	5	0.043
Valine[2]	57^{a}	67^{a}	57^{a}	73^{a}	81^{a}	151^{ab}	324^{b}	444^{c}	583^{c}	24	0.0001
Alanine	974	942	1081	1114	1178	1092	940	968	968	109	NS
Arginine[2]	248^{a}	188^{a}	140^{a}	162^{a}	119^{b}	140^{a}	167^{a}	124^{b}	154^{a}	16	0.028
Aspartate[2]	533^{a}	545^{a}	479^{a}	339^{a}	221^{ab}	109^{ab}	83^{b}	62^{b}	41^{b}	22	0.0001
Citrulline[2]	274^{a}	213^{a}	220^{a}	153^{a}	164^{a}	84^{b}	87^{b}	65^{b}	70^{b}	19	0.0001
Cystine	1	3	5	2	5	3	3	4	2	1	NS
Glutamate	438	404	369	388	267	230	202	194	204	44	NS
Glutamine[2]	545^{a}	565^{a}	296^{ab}	384^{ab}	168^{b}	61^{c}	69^{c}	46^{c}	52^{c}	29	0.0001
Glycine[2]	2406^{a}	2357^{a}	2144^{a}	1957^{a}	1245^{b}	1789^{a}	1494^{b}	1375^{b}	1788^{a}	281	0.005
Hydroxyproline	175	150	204	163	123	104	117	114	104	18	NS
Ornithine	286	245	236	203	190	145	134	147	141	18	NS
Proline	1288	1028	839	959	771	740	865	729	787	89	NS
Serine[2]	1090^{a}	1286^{a}	1112^{a}	1101^{a}	789^{ab}	505^{b}	482^{b}	399^{c}	422^{c}	94	0.0001
Taurine	297	302	267	281	375	196	220	216	244	21	NS
Tyrosine[2]	328^{a}	323^{a}	254^{ab}	239^{ab}	150^{b}	73^{c}	87^{c}	75^{c}	67^{c}	12	0.0027

[1]Values represent the means; n=4

[2] Overall ANOVA, F-test:P<0.05. Values with different superscript letters are significantly different (Tukey's multiple comparisons)

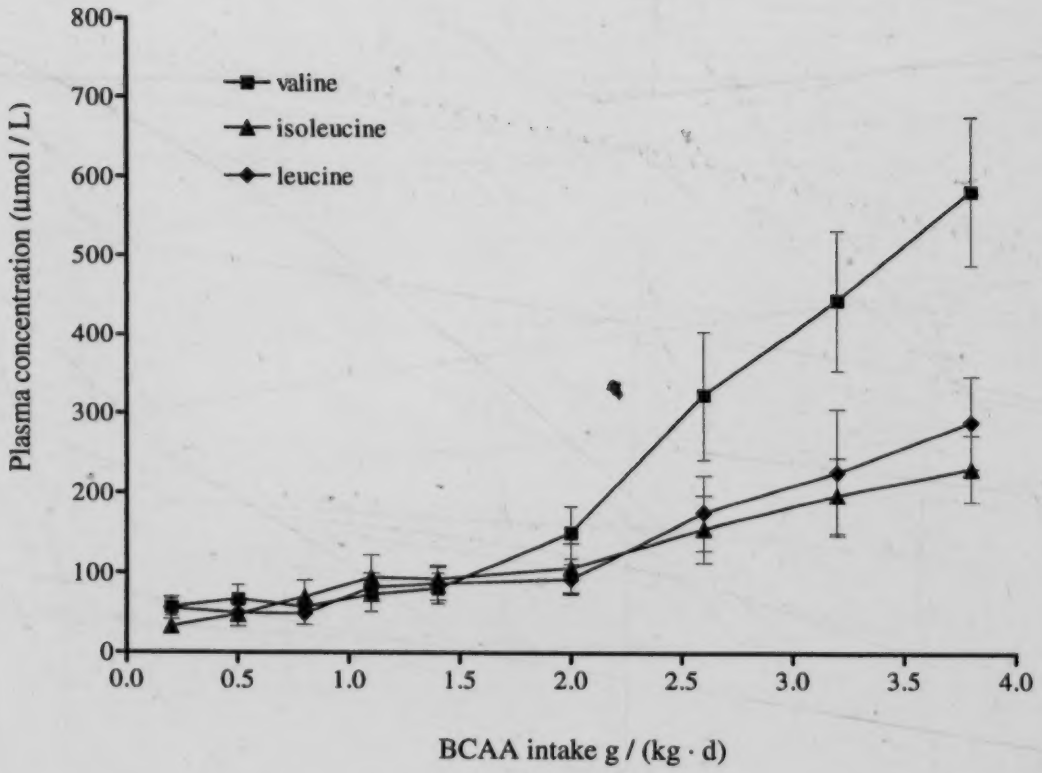

Figure 3.2 Mean BCAA concentrations in plasma of parenterally fed piglets receiving graded intakes of BCAA (n = 4 per treatment, error bars represent 1 SD)

3.3.2 ***Enteral BCAA requirement:*** Phenylalanine flux (198.1 μmol/(kg · h), pooled SE: 24.3) and intake (114.1 μmol/(kg · h), pooled SE: 7.6), were not significantly different ($P>0.05$) across diet treatments, similar to experiment 1, and as required by the IAAO technique. Phenylalanine oxidation was significantly influenced by BCAA intake expressed as a percentage of the dose oxidized (**Figure 3.3**). As the total BCAA intake increased from 0.2 to 2.6 g/(kg · d), phenylalanine oxidation decreased significantly ($P<0.05$). Further increases in BCAA intake from 2.6 to 3.8 g/(kg · d), did not significantly affect phenylalanine oxidation ($P>0.05$, slope not different from zero).

As in experiment 1, aspartate, serine and glycine, which were used to make the diets iso-nitrogenous, had higher plasma concentrations in the BCAA deficient diets and their concentrations decreased significantly with increases in BCAA intake (**Table 3.2**). Plasma concentrations of several amino acids (glutamate, glutamine, taurine, histidine, tyrosine, proline, ornithine and lysine) decreased as the supply of the limiting amino acids increased (Table 3.2). BCAA intake, as in experiment 1, had a significant effect on plasma phenylalanine concentration, which decreased from 134 to 84 μmol / L as BCAA intake increased from 0.2 to 2.6 g/(kg · d), respectively ($P<0.05$); no significant changes in plasma phenylalanine concentrations were observed with further increases in BCAA intake. The plasma concentrations of leucine remained low (~50 μmol / L) (**Figure 3. 4**), and did not vary significantly with increases in BCAA intake from 0.2 to 2.0 g/(kg · d). As BCAA intake increased from 2.6 to 3.8 g/(kg · d) the concentration of leucine increased significantly (106 to 263 μmol / L, respectively). Plasma isoleucine and valine concentrations followed a markedly different pattern (Figure 3.4)

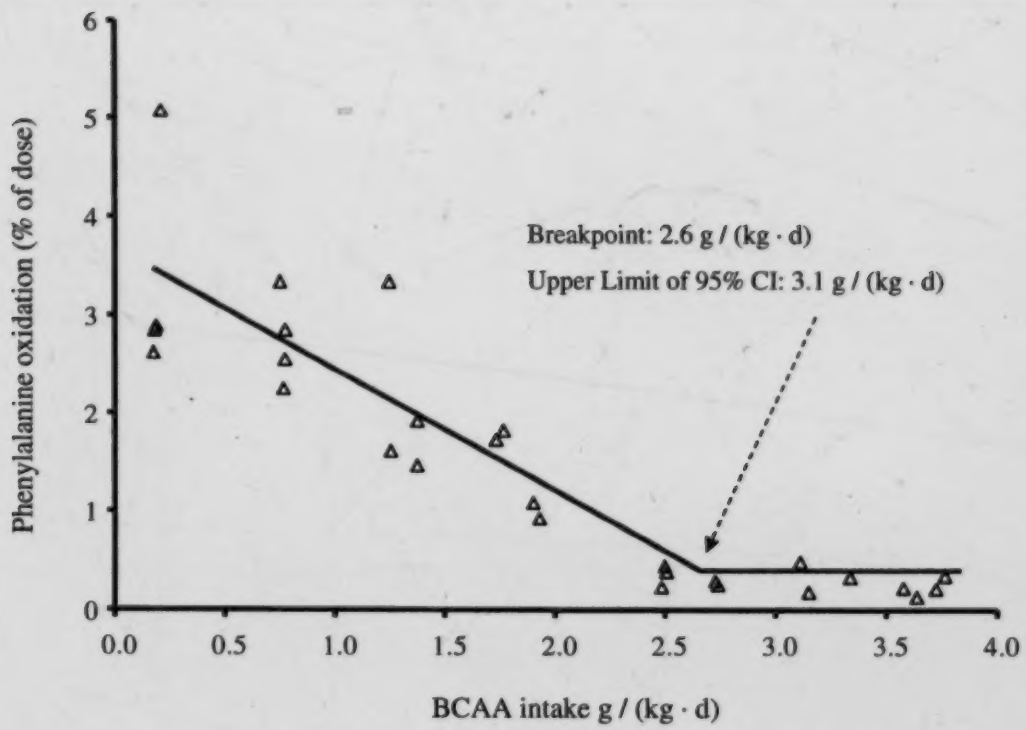

Figure 3.3 Oxidation of L-[1-^{14}C]phenylalanine as a percentage of dose in enterally fed piglets receiving graded intakes of BCAA (n=28)

Table 3.2 Plasma amino acid concentrations of enterally fed piglets receiving graded intakes of BCAA[1]

Amino acid (μ mol / L)	BCAA intake g / (kg · d) 0.2 (3)[1]	0.8 (4)[1]	1.4 (4)[1]	2.0 (4)[1]	2.6 (3)[1]	3.2 (3)[1]	3.8 (4)[1]	SE	ANOVA p value
Histidine[2]	80[b]	135[a]	59[b]	63[b]	37[b]	50[b]	47[b]	9	0.0001
Isoleucine[2]	118[a]	274[a]	638[ab]	655[b]	488[b]	492[b]	404[b]	26	0.0001
Leucine[2]	13[a]	11[a]	16[a]	22[a]	106[b]	255[c]	263[c]	7	0.0001
Lysine[2]	1462[a]	1370[a]	1038[a]	853[b]	760[b]	996[b]	754[b]	155	0.039
Methionine	32	35	37	30	30	26	23	2	NS
Phenylalanine[2]	127[a]	134[a]	121[a]	85[ab]	84[ab]	77[b]	65[b]	12	0.0084
Threonine	1385	1844	1600	1378	1166	1402	916	173	NS
Tryptophan	51	47	50	44	56	60	43	7	NS
Valine[2]	220[a]	341[a]	855[b]	1133[c]	1025[c]	1163[c]	1103[c]	37	0.0001
Alanine	1767	1954	1967	2092	1520	1652	1055	264	NS
Arginine	240	263	223	206	260	200	199	21	NS
Aspartate[2]	113[a]	134[a]	59[b]	46[bc]	24[bc]	20[bc]	12[c]	6	0.0001
Citrulline[2]	256[a]	244[a]	209[ab]	145[ab]	160[ab]	118[b]	120[b]	21	0.0029
Cystine	5	3	3	2	6	2	3	1	NS
Glutamate[2]	736[a]	781[a]	596[b]	475[b]	292[d]	359[b]	254[d]	34	0.0001
Glutamine[2]	562[a]	502[a]	361[a]	199[ab]	87[b]	75[b]	64[b]	28	0.0001
Glycine[2]	2567[a]	2696[a]	2300[a]	2210[a]	1923[ab]	1656[ab]	930[c]	215	0.0009
Hydroxyproline	109	132	118	151	137	155	137	13	NS
Ornithine[2]	157[ab]	284[a]	249[a]	155[ab]	170[ab]	164[ab]	133[b]	26	0.009
Proline[2]	1377[a]	1747[a]	1347[a]	980[b]	1007[a]	861[b]	646[b]	161	0.0104
Serine[2]	1438[a]	1591[a]	1134[a]	865	443[c]	398[c]	222[d]	110	0.0001
Taurine[2]	489[a]	487[a]	367[ab]	243[b]	211[b]	170[bc]	159[bc]	30	0.0001
Tyrosine[2]	198[a]	229[a]	157[a]	114[ab]	70[b]	71[b]	49[b]	15	0.0001

[1] Values represent means; the number of pigs is given in parentheses in the column heading

[2] Overall ANOVA, F-test:$P<0.05$. Values with different superscript letters are significantly different (Tukey's multiple comparisons)

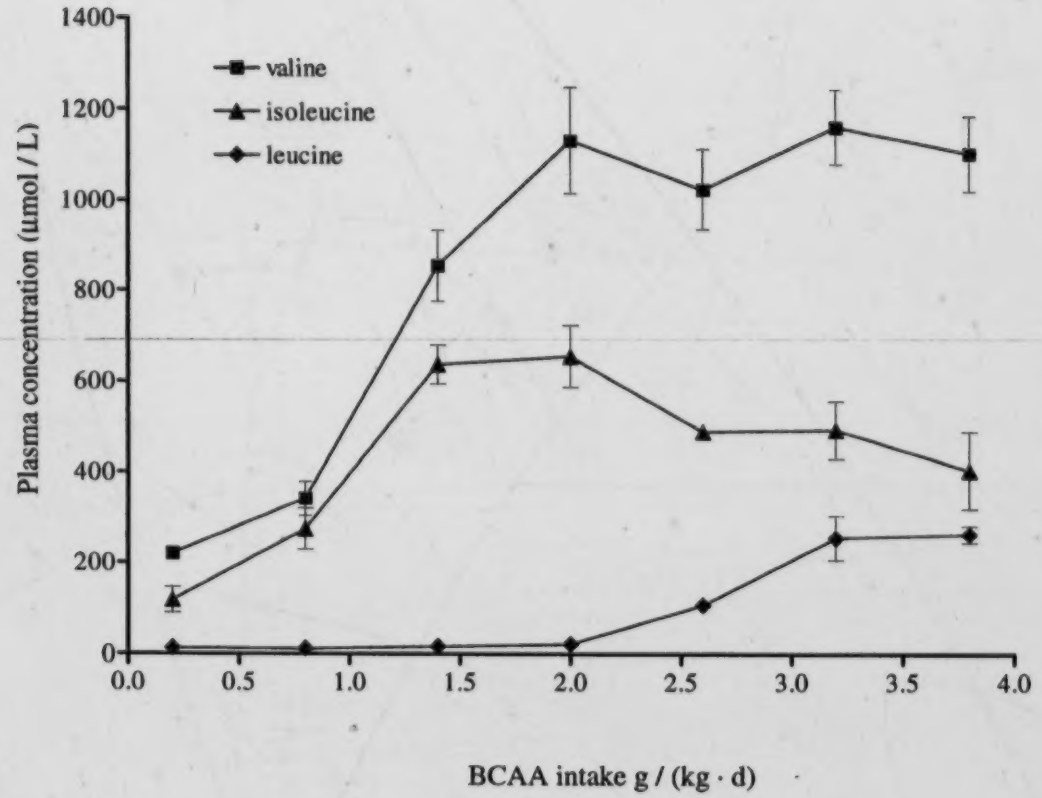

Figure 3.4 Mean BCAA concentrations in plasma of enterally fed piglets receiving graded intakes of BCAA (n = 4 per treatment, error bars represent 1 SD)

than in experiment 1 (Figure 3.2) beginning at the lowest BCAA intake of 0.2 g/(kg · d). Plasma isoleucine and valine concentrations increased significantly until the total BCAA intake was 2.0 g/(kg · d). With further increases in BCAA intake [2.6 to 3.8 g/(kg · d)], plasma isoleucine concentration decreased, although not significantly ($P>0.05$) and valine concentration remained high.

To determine the total BCAA requirement in both parenterally and enterally fed piglets, breakpoint analysis was conducted on the data points using a combined two-phase linear regression model (Figures 3.1 and 3.3). The data partitioning was based on the model which produced the lowest combined sum of squares for error. The breakpoint estimate for phenylalanine oxidation as a percentage of dose in parenterally fed piglets was determined to be 1.53 g/(kg · d), 95 % CI: 1.07 – 1.99 (Figure 3.1). In the case of enterally fed piglets, the breakpoint analysis yielded estimates of 2.64 g/(kg · d), 95 % CI: 2.15 – 3.13 (Figure 3.3) for phenylalanine oxidation as a percentage of dose.

3.4 Discussion

The piglet model of TPN feeding developed in our group, and the indicator amino acid oxidation technique has been successfully used to determine many amino acid requirements (Brunton et al, 1998). The requirements for several amino acids differ between routes of parenteral or enteral feeding (Bertolo et al, 1998 and Brunton et al, 1999) and there is increasing evidence that currently available commercial amino acid solutions for nutritional support are inappropriate for maximizing nitrogen efficiency

(Brunton et al, 2000). Estimation of the parenteral amino acid requirements from the requirements for oral feeding has several disadvantages, as parenteral feeding bypasses the gut and results in lower total metabolic mass of the gut (Ball et al, 1996, Brunton et al, 2000 and Bertolo et al, 1998). Thus, in order to provide a direct measurement of the parenteral BCAA requirement, in this study, piglets were supplied with an identical diet enterally and parenterally and the BCAA requirements were determined.

The mean parenteral BCAA requirement, as determined by the breakpoint of the two-phase regression crossover model, was found to be 1.53 g/(kg · d) (Figure 3.1) when based on phenylalanine oxidation as a percentage of dose. The mean enteral requirement was estimated to be 2.64 g/(kg · d) (Figure 3.3), based on phenylalanine oxidation as a percentage of dose. The safe level of BCAA intake, which would meet the needs of 95% of the population, or the upper 95% CI of the breakpoint estimate in parenteral feeding is recommended as 1.99 g/(kg · d); and in enteral feeding as 3.13 g/(kg · d).

These results, suggesting that the small intestine utilizes BCAA to a large extent (~44%of intake) is significant because BCAA catabolism has been viewed to be mainly carried out in extrahepatic tissues, due to the higher activity of branched chain amino transferase (BCAT), the first enzyme in the catabolic pathway of the BCAA, in skeletal muscle and the relatively lower activity of BCAT in the liver (DeSantiago et al, 1998). The indicator amino acid oxidation method measures whole body utilization of BCAA for protein synthesis; therefore the results obtained include the metabolism and utilization of branched chain alpha ketoacids (BCKA). Stoll et al (1998) measured appearance of amino acids in portal blood in 28 d old piglets, fed sow milk replacer

continuously via catheters. They reported 57%, 61% and 69% appearance of leucine, valine and isoleucine respectively in the portal blood, suggesting a portal drained visceral uptake of 43%, 39% and 31% of leucine, valine and isoleucine, respectively. Gelfand et al (1986) in adult human subjects observed one third of orally infused BCAA to be extracted by splanchnic tissues. Gelfand et al (1986) also suggested that the earlier observations (Felig, 1976 and Wahren et al, 1976) of BCAA selectively escaping the splanchnic bed after a protein meal led researchers to underemphasize the importance of splanchnic tissues in BCAA catabolism. These values (Gelfand et al, 1986 and Stoll et al, 1998) compare well with the 44% extraction of total BCAA, as observed in the current study.

In both the current experiments, BCAA were provided in the diets at a fixed ratio of 1:1.8:1.2 (isoleucine/leucine/valine), in order to remove the potential for antagonism occurring among the BCAA and affecting the requirement values. To the authors knowledge this is the first time such an approach has been used to determine BCAA requirements. The advantage of this approach is that it avoids the possible effects of antagonism on the requirements for the individual amino acids and simultaneously provides a test of whether or not the ratio of the BCAA is optimal.

In parenterally fed piglets, the plasma concentrations of BCAA, remained low until the BCAA intake reached 1.5 g/(kg · d) (Figure 3.2). The similarity in responses for the three BCAA when fed below the requirement suggests that the ratio used was close to optimal. All three BCAA concentrations continued to increase with increasing intakes of BCAA above requirement, but the different slope of the response for valine after BCAA intakes of 1.5 g/(kg · d), suggests that it is being metabolized

differently than leucine and isoleucine. An increasing rate of accumulation of valine in the plasma compared to leucine and isoleucine, suggests a lower relative rate of catabolism of valine once requirement has been met. The BCAA share a common transport system into the cells; the Large Neutral Amino Acid (LNAA) carrier system or the L system (Skeie et al, 1990). Competition for uptake into the cells among the BCAA may play a role in the increased rate of accumulation of valine in plasma, when BCAA are provided in a fixed ratio at levels higher than the total BCAA requirement.

The plasma concentrations of BCAA in enterally fed piglets (Figure 3.4) provided even more interesting results. Leucine followed the expected pattern; plasma concentrations of the limiting amino acid remained low until requirement was reached, and once the requirement was met, the limiting amino acid concentrations started to increase. On the contrary, the plasma concentrations of isoleucine and valine were high (118 μ mol / L and 220 μ mol / L, respectively, Table 3.2) even when the supply of total BCAA in the diet was most deficient (0.2 g/(kg · d)). The concentrations of isoleucine and valine continued to increase with increasing supply of total BCAA, and valine concentrations appeared to reach a plateau once the total BCAA requirement was reached. The concentration of isoleucine showed a decreasing trend (not statistically significant, $P>0.05$), once total BCAA requirement was reached. These responses could be partly a result of the presence of BCAA catabolizing enzymes in the gut of the piglet. Although the enzymes have been shown to be present in the gut of rat and in humans (Suryawan et al, 1998), to the authors knowledge there is no comparable data in the pig. Similar patterns in plasma BCAA concentrations have been previously observed in pigs (Mitchell et al, 1968) and in other species; human infants (Snyderman et al, 1968),

human adults (Hambraeus et al, 1976), rats (Clark et al, 1976) and kittens (Hargrove et al, 1984). In these experiments all subjects were fed enterally and a dietary deficiency of leucine was concluded to cause elevated plasma concentrations of isoleucine and valine. In the current experiments, the previously described pattern was observed in enterally fed piglets (Figure 3.4); but not in parenterally fed piglets (Figure 3.2), in spite of the fact that both routes of feeding had identical amino acid intakes. We speculate that the differences in plasma amino acid pattern indicates that not only does the total requirement for BCAA differ between parenteral and enteral feeding, but that the optimum ratio of BCAA also differs between the routes of feeding. Thus, further experiments need to be conducted to determine the optimum ratio of the BCAA during both parenteral and enteral feeding.

The pattern of BCAA in the plasma of enterally fed piglets, when compared with the parenterally fed piglets clearly demonstrates that the gut has a high demand for leucine and a clear preference for leucine compared to isoleucine or valine. If the gut was utilizing all three BCAA in the same proportion as the rest of the body, then the plasma amino acid pattern would have been similar for both routes of feeding. The observation, during enteral feeding, that valine and isoleucine increased in plasma while leucine remained low indicates that leucine is being extracted by the gut and therefore may be limiting protein synthesis in the rest of the body. Valine and isoleucine are not being utilized by the gut to the same extent and are being passed to the systemic circulation, but because protein synthesis is limited by leucine, these two amino acids increase in concentration in the plasma. Examination of Table 3.2 shows that when

leucine is fed below requirement, and thus limiting protein synthesis, most of the other essential amino acids are higher in plasma, than when leucine is fed above requirement.

Yu et al (1990) in a quantitative determination of leucine extraction by the splanchnic tissues in 20-25 kg dogs, suggested that 30 –35% of total ingested leucine was being metabolized by the gut tissues. Thus, it will be important to quantitate and determine the fate of the extracted leucine in neonatal piglets. This will provide important information regarding a potential role of BCAA in the development and metabolism of the intestine, especially in low birth weight infants.

Similar to the current study, the concentration of valine in plasma in suckling piglets, and human infants (**Table 3.3**), were relatively higher than isoleucine and leucine. As discussed earlier, this increased accumulation may be due to competition for uptake of valine into cells once the total BCAA requirement has been met. The pattern of BCAA in human and sow milk (1:1.8:1 and 1:2.4:1.4 respectively of ile/leu/val) closely resembles the pattern seen in the respective human fetal and piglet tissues (Table 3.3). Although this pattern may be appropriate in the case of orally fed piglets and human infants, the optimum ratio for parenteral solutions is yet unknown. Commercially available parenteral solutions today display a wide variation in the ratio among the BCAA (Brunton et al, 2000) and increases the chances of either underfeeding or overfeeding of any one amino acid. This could potentially be a cause for antagonism occurring between the BCAA and thereby placing the neonate at risk. Based on the current observations on plasma BCAA patterns due to different routes of feeding, it is important that the appropriate ratio of BCAA for the neonate be determined both parenterally and enterally.

Table 3.3 Comparison of BCAA concentrations in piglet plasma and human infant plasma, human and piglet milk and fetal tissues

Amino acid	Suckling piglets[1]	Enterally fed piglets[2]	Parenterally fed piglets[2]	Breast fed infants[3]	Parenterally fed preterm infants[4]	Human milk[5]	Sow milk[5]	Human fetal tissue[6]	Piglet fetal tissue[7]
			(μ mol / L)				(g / 100g	total amino	acids)
Isoleucine	135	120	110	56	70	5.9	3.6	3.5	3.0
Leucine	195	272	258	101	123	10.8	8.8	7.5	7.0
Valine	318	248	250	150	169	6.1	5.0	4.7	4.4
Ratio (Ile/Leu/Val)	1:1.4:2.4	1:2.3:2.1	1:2.4:2.3	1:1.8:2.7	1:1.8:2.4	1:1.8:1	1:2.4:1.4	1:2.1:1.3	1:2.3:1.5

[1] Seven day old suckling piglets, Flynn et al (2000)

[2] Seven day old piglets fed elemental diet enterally or parenterally, Bertolo et al, (2000)

[3] Three month old breast fed infants, Akeson et al (1998)

[4] Preterm infants fed TrophAmine (Kendall-McGraw Laboratories, Irvine, CA) parenterally for seven consecutive days, Adamkin et al (1991)

[5] Davis et al (1993)

[6] Widdowson (1979)

[7] Wu et al (1999)

In conclusion, a mean total BCAA requirement for neonatal piglets was determined to be 1.53 g/(kg · d) during parenteral feeding, compared to 2.64 g/(kg · d) for enteral feeding, when a fixed ratio of BCAA (1:1.8:1.2; isoleucine/leucine/valine) was provided. The uptake of 44% of enterally fed BCAA by the splanchnic tissues during first pass intestinal metabolism is a significant finding, because the BCAA are generally accepted to be predominantly metabolized by the extra hepatic tissues. The different responses in plasma concentration of the BCAA during parenteral and enteral feeding routes suggests that the optimum ratio of BCAA will be different and specific for each route of nutrient delivery and the optimum ratio needs to be determined experimentally.

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4.0 PARENTERAL AND ENTERAL ROUTES OF FEEDING IN NEONATAL PIGLETS REQUIRE DIFFERENT RATIOS OF BRANCHED-CHAIN AMINO ACIDS

4.1 Introduction

Previously we determined the total branched-chain amino acid (BCAA; isoleucine, leucine and valine) requirement during parenteral and enteral routes of feeding in neonatal piglets (Elango et al, 2002: Chapter 3). The mean total BCAA requirement was determined to be 1.53 g/(kg · d) during parenteral feeding, compared with 2.64 g/(kg · d) for enteral feeding, when a fixed ratio of BCAA (1:1.8:1.2;isoleucine/leucine/valine) was provided (Elango et al, 2002: Chapter 3). The ratio among BCAA used in the previous study was based on the NRC requirements for swine (1998), for piglets weighing between 1 and 5 kg. The NRC recommendations were based on oral feeding studies and therefore may not be appropriate for different routes of feeding.

Feeding by total parenteral nutrition (TPN) bypasses first-pass metabolism by the small intestine and liver, and therefore nutrients are provided to the peripheral organs in different concentrations when compared to enteral feeding. Thus, route of feeding alters whole-body nitrogen metabolism (Duffy & Pencharz, 1986) and, organ and plasma amino acid concentrations (Bertolo et al, 2000). Premature neonates and low-birth-weight (LBW) infants often receive TPN as an essential component of nutritional support and evidence is accumulating that current TPN solutions are inadequate (Brunton et al, 2000). The previous study (Elango et al, 2002: Chapter 3) showing total BCAA

requirement being significantly different between parenteral and enteral nutrition further strengthens this evidence and the optimum ratio of BCAA might also be different and specific for each route of nutrient supply, however this has not been investigated to the authors' knowledge.

BCAA enriched TPN and enteral nutrition have been used as therapeutic agents in various catabolic states such as hepatic encephalopathy, chronic renal failure, muscle protein wasting, trauma and sepsis (Lal & Chugh, 1995). In premature neonates, BCAA enriched TPN was shown to decrease apnea and improve respiratory pattern and function (Blazer et al, 1994). In spite of the numerous studies conducted on BCAA supplementation, clinical results have remained inconclusive and controversial (Teasley & Buse, 1989 and Vente et al, 1991). One of the reasons for such varied outcomes of BCAA supplementation could be due to an inappropriate ratio among BCAA being used in TPN and enteral nutrition.

BCAA have been shown to exhibit antagonism whereby excessive intakes of any one BCAA, especially leucine, will have an impact on the fate and utilization of the other two BCAA, resulting in altered blood and tissue amino acid concentrations (Block, 1989). Antagonism among BCAA results in severe growth impairment and food intake depression in young growing animals (Harper et al, 1984). Thus, determining the optimum ratio among BCAA is crucial to achieve our long term goal of defining the "optimal" profile for neonatal TPN which provides all amino acids in the balance necessary to maximize protein synthesis and with minimal excess.

In the present study our, objective was to determine whether the optimum ratio among BCAA would differ between parenteral and enteral routes of feeding in

neonatal piglets using the indicator amino acid oxidation (IAAO) method. Change in oxidation of phenylalanine was measured following supplementation of isoleucine, leucine or valine, to meet 100% of requirement to a diet which provided 75% of total BCAA requirement for parenteral and enteral routes respectively (as determined previously, 1). Addition of the most limiting amino acid among the three BCAA was hypothesized to significantly decrease the oxidation of the indicator amino acid, phenylalanine.

4.2 Materials and Methods

4.2.1 ***Animals and study protocol:*** The Animal Care Committee of the University of Alberta approved all procedures used in this experiment. Male Yorkshire piglets (n = 24), weighing approximately 1.5 kg and, 1 - 2 d old were transferred to the Metabolic Research Facility at the University of Alberta. The piglets were weighed and anaesthetized (0.8% halothane) for the surgical implantation of catheters (Ed-Art, Don Mills, Canada). Venous catheters were placed following modified procedures of Wykes et al. (1993) and Rombeau et al. (1984) for gastric catheters. The surgical procedures were recently described by Bertolo et al. (1998). After surgery, the animals were fitted with adjustable cotton jackets, which prevent tangling and occlusion of the catheters. The laboratory conditions and piglet housing were described previously (Wykes et al, 1993).

4.2.2 ***Diet regimen:*** The composition of the elemental and complete diet was based on the initial formulation by Wykes et al. (1993), with modifications (Chapter 8, Table 8.1.1 and 8.1.2). Diet was infused (continuous, 24h) using infusion pumps via a

tether-swivel system (Alice King Chatham Medical Arts, Los Angeles, CA) intravenously (Experiment 1, parenteral BCAA ratio) or intragastrically (Experiment 2, enteral BCAA ratio). At full infusion rate [272 mL/(kg · d)], the complete diet provided 1.1 MJ available energy /(kg · d) and 15.3, 27.4, 9.4 g of amino acids, glucose and fat / (kg body weight · day), respectively. The base amino acid profile of the complete diet was (mg/g of total L-amino acids): alanine, 104; arginine, 78; aspartate, 60; cysteine, 14; glutamate, 103; glycine, 24; histidine, 31; isoleucine, 45; leucine, 103; lysine, 56; methionine, 19; phenylalanine, 40; proline, 82; serine, 32; taurine, 5; threonine, 52; tryptophan, 21; tyrosine, 27; valine, 52. The addition of individual BCAA to make the test diets is described below. The phenylalanine concentration of the diet was increased compared to that used previously (Elango et al, 2002: Chapter 3) to obtain a larger difference in oxidation between the dietary treatments, based on preliminary data. All piglets were administered the complete diet intravenously, at 50% of the full rate for approximately 6h after surgery, then at 75% overnight, for experiment 1, the diet was then infused at full rate intravenously. Whereas for experiment 2, the piglets were switched to 50 % of full rate intragastric feeding on d 1; and increased to the full intragastric feeding by d 2.

4.2.3 ***Test diets:*** On d 5, all piglets received diets that provided total BCAA at 75% of parenteral [1.1g/(kg · d)] and enteral [2.0 g/(kg · d)] requirement, as determined previously by IAAO studies (Elango et al, 2002: Chapter 3). The ratio among the three BCAA was 1:1.8:1.2, (isoleucine/leucine/valine) based on NRC recommendations (1998). This ratio is also very similar to that found in milk protein (Elango et al, 2002: Chapter 3). An oxidation study was conducted on d6 and the piglets

were returned to the complete diet for 24 h. At 2100h, on d 7 the piglets were randomly assigned to one of the 3 test diets [+ isoleucine, +leucine, +valine] (**Table 4.1**), which provided isoleucine, leucine or valine at 100% of requirement. The 100% requirement value was 1.53 g/ (kg · d) for experiment 1, and 2.64 g/ (kg · d) for experiment 2, as previously determined (Elango et al, 2002: Chapter 3). L-aspartate, L-serine and L-glycine were used to make the test diets isonitrogenous.

4.2.4 *Tracer infusion, sample collection and analytical procedures:*

Details of the infusion protocol, $^{14}CO_2$ and blood collection have been described previously (House et al, 1997). Briefly, on d 6 and 8, the piglets were transferred to plexiglass boxes, approximately 16 – 18 h after the start of the test diet infusion. A 30 min period was allowed for the piglets to acclimatize and the CO_2 to equilibrate in the chamber; following which phenylalanine flux and oxidation were determined by a primed [186 kBq (5 μCi) / kg], constant infusion [130 kBq (3.5 μCi) / (kg · h)] of a tracer solution containing 92.5 MBq (2.5 mCi) / L of L – [1-^{14}C]phenylalanine (American Radiolabeled Chemicals, Inc. St. Louis, MO) . Air was drawn from the boxes by a pump and the total amount of $^{14}CO_2$ expired was trapped in a series of gas washing bottles containing CO_2 absorber (ethanolamine and ethylene glycol monomethylether, 1:2, v/v). Blood samples (1.5 mL) were drawn at time 0 and every half h during the 4 h study. The blood samples were centrifuged (3000 x g for 5 min), and plasma collected and stored at -80°C, until analysis of phenylalanine specific radioactivity (SRA) and amino acid concentrations. On d 8, a 5-h study was conducted; h 1 was used for breath collection to determine baseline radioactivity, prior to the primed, constant infusion. Immediately

Table 4.1 Branched-chain amino acid intake of parenterally and enterally fed piglets

Amino Acid	*Experiment 1* Parenteral BCAA ratio				*Experiment 2* Enteral BCAA ratio			
	75% BCAA[1]	+isoleucine[2]	+leucine[2]	+valine[2]	75% BCAA[1]	+isoleucine[2]	+leucine[2]	+valine[2]
	g/(kg · d)							
Isoleucine	0.275	0.705	0.275	0.275	0.5	1.14	0.50	0.50
Leucine	0.495	0.495	0.925	0.495	0.9	0.90	1.54	0.90
Valine	0.330	0.330	0.330	0.760	0.6	0.60	0.60	1.24
Total BCAA	1.1	1.53	1.53	1.53	2.0	2.64	2.64	2.64
Ratio (ile/leu/val)	1:1.8:1.2	2.1:1.5:1	1:3.4:1.2	1:1.8:2.8	1:1.8:1.2	1.9:1.5:1	1:3.1:1.2	1:1.8:2.5

[1]75% BCAA diet at a total BCAA intake of 1.1 g/(kg · d) and 2.0 g/(kg · d), respectively for parenteral and enteral feeding, at a fixed ratio of 1:1.8:1.2; isoleucine/leucine/valine

[2]Test diets where either isoleucine, leucine or valine was supplemented to meet 100% of total BCAA requirement; 1.53 g/(kg · d) and 2.64 g/(kg · d) for parenteral and enteral requirement, respectively.

upon completion of the oxidation study on d 8, the piglets were injected with a lethal dose (750mg) of sodium pentobarbital through the venous sampling line.

The rate of expiration of $^{14}CO_2$ was determined by liquid scintillation counting of radioactivity in the CO_2 absorber. The plasma concentrations of amino acids and SRA of plasma phenylalanine were analyzed by reverse-phase high performance liquid chromatography (HPLC); collection and liquid scintillation counting of radioactive fractions were described previously (House et al, 1997). Phenylalanine intake, flux, percentage of dose oxidized and oxidation were calculated as described previously (House et al, 1997).

4.2.5 ***Statistical analyses:*** Each experiment was a fully randomized design. Phenylalanine kinetics and plasma concentrations were analyzed as a one-way ANOVA with diet as the main effect within each route of feeding (n=12). Differences between d 6 and d 8 for oxidation of L – [1-^{14}C]phenylalanine as a % of dose oxidized was analyzed as a 2 x 3 factorial ANOVA with interaction (n=24), where factor 1 was the route of feeding (parenteral or enteral) and factor 2 was the test diet (+isoleucine, +leucine or +valine). The PROC GLM procedure was employed (SAS/STAT version 8.01, SAS institute, Cary, NC) and least square means were considered significant at $P < 0.05$.

4.3 Results

All piglets remained healthy, active and interested in the environment through the entire course of both the experiments. The initial weights (1.58 ± 0.16 kg), weight at study (2.61 ± 0.29 kg) and average daily weight gain (149 and 157g/d in

experiments 1 and 2, respectively), were not significantly (P>0.05) different among diet treatments or between routes of feeding.

4.3.1 ***Parenteral BCAA ratio:*** Phenylalanine flux [330.4 μmol/(kg · h), SEM: 42.3] and phenylalanne intake [121 μmol/(kg · h), SEM: 2.9], were not significantly (P > 0.05) different between 75% BCAA diets and test diets. Supplementation of isoleucine, (+isoleucine), leucine (+leucine) or valine (+valine) to the parenteral 75% BCAA diet decreased phenylalanine oxidation, as a percentage of dose oxidized, calculated within individual pigs (**Figures 4.1, 4.2 and 4.3**). The percentage decrease (Δ) in oxidation within pigs fed diets providing 75% of parenteral BCAA requirement compared to +isoleucine and +valine diets was 12.6% and 6.6% respectively (P < 0.05) (**Figure 4.4**). Supplementation of leucine (+leucine), did not significantly (P > 0.05) affect phenylalanine oxidation (~2%) (Figure 4.4).

The supplementation of isoleucine increased plasma concentrations of isoleucine, leucine and threonine significantly (P < 0.05), compared to the 75% BCAA diet but had no effect on valine (**Table 4.2**). The +leucine diet did not affect plasma BCAA concentrations (Table 4.2). The +valine diet significantly (P < 0.05) increased plasma valine concentrations compared to the 75% BCAA diet, but did not affect the other two BCAA (Table 4.2). Supplementation of isoleucine, leucine or valine to the 75% BCAA diet did not affect plasma concentrations of dietary dispensable amino acids (data not shown).

4.3.2 ***Enteral BCAA ratio:*** Phenylalanine flux [412.8 μmol/(kg · h), SEM: 22.6] and phenylalanine intake [122 μmol/(kg · h), SEM: 3.3], were not significantly (P > 0.05) different between 75% BCAA diets and supplemented diets. Phenylalanine

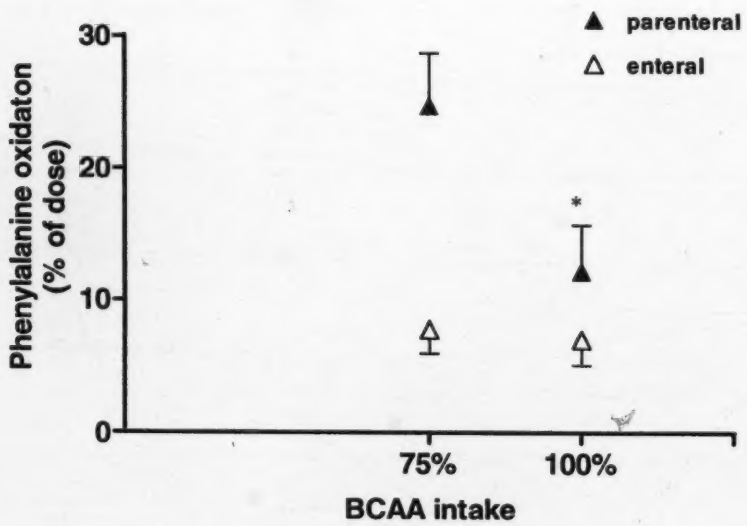

Figure 4.1 Effect of supplementing Isoleucine to 75% Branched-chain amino acid diet on oxidation of L-[1-^{14}C]phenylalanine (percentage of dose) in parenterally (*expt.1*) and enterally (*expt.2*) fed piglets. *Addition of isoleucine in parenterally fed piglets decreased oxidation of phenylalanine (%of dose) significantly (ANOVA $P < 0.05$) (n = 4 per treatment, values are means ± SEM)

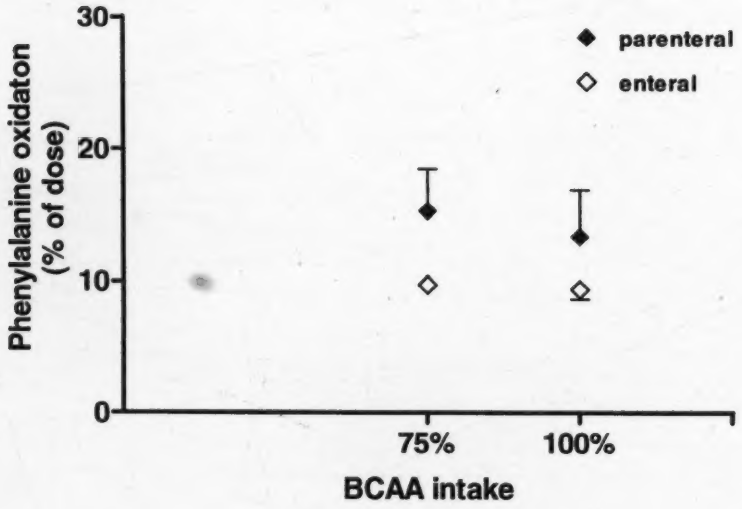

Figure 4.2 Effect of supplementing Leucine to 75% Branched-chain amino acid diet on oxidation of L-[1-^{14}C]phenylalanine (percentage of dose) in parenterally (*expt. 1*) and enterally (*expt. 2*) fed piglets. No significant changes to addition of Leucine (ANOVA $P > 0.05$) (n = 4 per treatment, values are means ± SEM; error bars for some means were too small to be displayed)

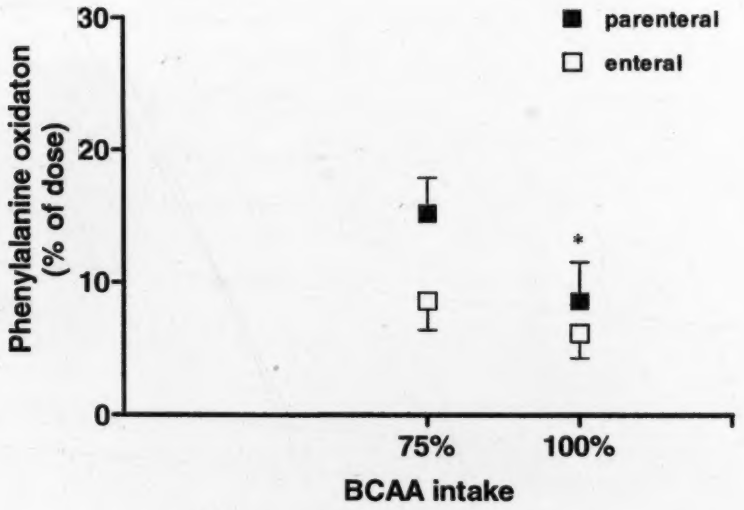

Figure 4.3 Effect of supplementing Valine to 75% Branched-chain amino acid diet on oxidation of L-[1-^{14}C]phenylalanine (percentage of dose) in parenterally (*expt. 1*) and enterally (*expt. 2*) fed piglets. *Addition of Valine in parenterally fed piglets decreased oxidation of phenylalanine (%of dose) significantly (ANOVA $P < 0.05$) (n = 4 per treatment, values are means ± SEM)

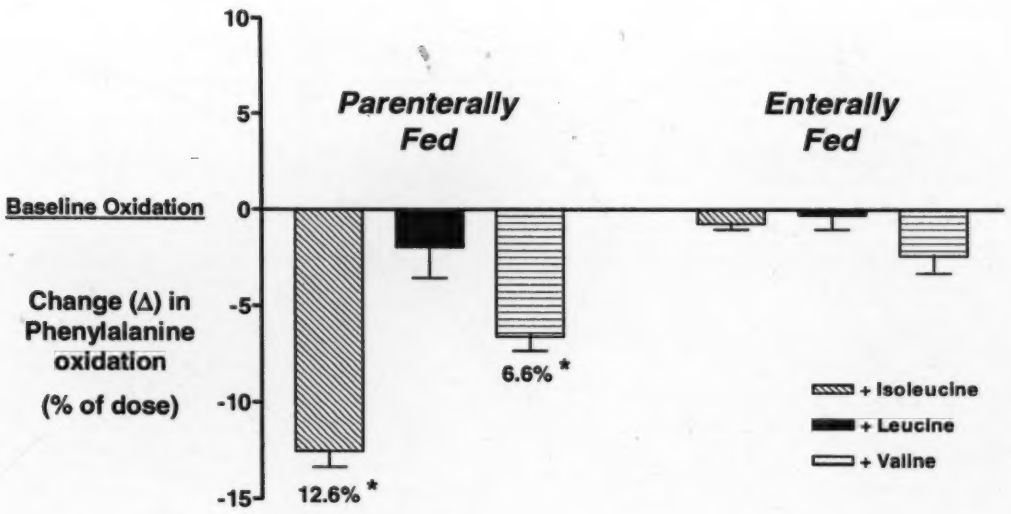

Figure 4.4 Mean change (Δ) for individual pigs in percentage dose oxidized of L-[1-^{14}C]phenylalanine due to supplementation of BCAA in parenterally and enterally fed neonatal piglets. * Supplementation of isoleucine and valine decreased phenylalanine oxidation (% of dose) by 12.6% and 6.6 % respectively, in parenterally fed piglets and were significant at $P < 0.05$. (n = 4 per treatment, error bars represent SEM)

Table 4.2 Changes in plasma free amino acid concentrations of parenterally fed piglets receiving a 75% BCAA diet followed by individual supplementation of isoleucine, leucine or valine *(expt.1)*[1]

Amino acid	75% BCAA	+ isoleucine	75% BCAA	+ leucine	75% BCAA	+ valine
			(μ mol/L)			
Histidine	84 ± 23	117 ± 65	63 ± 18	61 ± 37	95 ± 34	80 ± 27
Isoleucine	70 ± 13	385 ± 151**	49 ± 17	87 ± 42	68 ± 35	57 ± 22
Leucine	76 ± 40	353 ± 88**	58 ± 15	69 ± 13	61 ± 6	64 ± 19
Lysine	458 ± 50	371 ± 148	533 ± 211	364 ± 108	460 ± 103	382 ± 142
Methionine	16 ± 6	26 ± 4	14 ± 3	18 ± 3	18 ± 6	21 ± 11
Phenylalanine	113 ± 17	112 ± 20	101 ± 28	91 ± 18	101 ±16	109 ± 18
Threonine	667 ± 142	327 ± 108**	683 ± 181	346 ± 212	585 ± 59	543 ± 51
Tryptophan	66 ± 10	63 ± 9	61 ± 28	75 ± 21	49 ± 16	62 ± 11
Valine	95 ± 43	98 ± 37	38 ± 18	42 ± 9	76 ± 28	1572 ± 835**

[1]Values are means ± SD; n=4

** Significantly different from 75% BCAA diet (ANOVA: $P < 0.05$)

oxidation (percentage of dose oxidized) was not significantly ($P > 0.05$) affected by +isoleucine, +leucine or +valine (Figures 4.1, 4.2 and 4.3). When expressed as a percentage decrease (Δ) in oxidation from 75% BCAA diets (Figure 4.4), the +valine diet decreased phenylalanine oxidation by 2.4%, however this was not significant ($P = 0.08$) (Figure 4.3).

Changes in plasma BCAA concentrations were observed in the enterally fed piglets. The +isoleucine diet increased plasma isoleucine concentration significantly ($P < 0.05$) (**Table 4.3**), but had no significant ($P > 0.05$) effect on leucine and valine concentrations. For the +leucine diet, plasma isoleucine concentration decreased significantly ($P < 0.05$) and plasma leucine concentration increased significantly ($P < 0.05$). Plasma valine concentrations decreased, although not significantly. In general all three BCAA were within normal plasma concentrations for enterally fed piglets (1). In the +valine diet, plasma isoleucine concentration decreased significantly ($P < 0.05$) (Table 4.3). Leucine and valine plasma concentrations were not significantly ($P > 0.05$) affected. No changes were observed in the rest of the plasma amino acids.

4.4 Discussion

In the previous study (Elango et al, 2002: Chapter 3) we determined the total BCAA requirement in parenterally and enterally fed neonatal piglets using the indicator amino acid oxidation (IAAO) technique and found significant differences due to route of feeding. The parenteral requirement of total BCAA was ~56% of the enteral requirement. The ratio among the BCAA in the previous study was kept constant at 1:1.8:1.2 (isoleucine/leucine/valine), based on the NRC recommendations for swine

Table 4.3 Changes in plasma free amino acid concentrations of enterally fed piglets receiving a 75% BCAA diet followed by individual supplementation of isoleucine, leucine or valine *(expt. 2)*[1]

Amino acid	75% BCAA	+ isoleucine	75% BCAA	+ leucine	75% BCAA	+ valine
			(μmol/L)			
Histidine	58 ± 33	73 ± 18	69 ± 13	63 ± 4	36 ± 24	58 ± 18
Isoleucine	542 ± 136	955 ± 195**	626 ± 281	55 ± 11**	578 ± 142	195 ± 85**
Leucine	32 ± 12	54 ± 23	49 ± 18	109 ± 41**	34 ± 8	61 ± 27
Lysine	679 ± 83	585 ± 75	656 ± 168	559 ± 87	582 ± 196	573 ± 113
Methionine	20 ± 3	23 ± 6	25 ± 6	24 ± 9	16 ± 4	23 ± 7
Phenylalanine	91 ± 17	103 ± 8	108 ± 21	98 ± 20	98 ± 8	106 ± 17
Threonine	1016 ± 119	1197 ± 135	821 ± 167	880 ± 229	721 ± 159	1178 ± 245**
Tryptophan	43 ± 18	68 ± 23	49 ± 14	37 ± 11	61 ± 17	42 ± 12
Valine	918 ± 186	1022 ± 145	826 ± 79	571 ± 114	848 ± 172	1092 ± 349

[1]Values are means ± SD; n=4

** Significantly different from 75% BCAA diet (ANOVA: $P < 0.05$)

(1998). Although we supplied a constant ratio of BCAA in the diet, we observed marked differences in plasma concentrations of the BCAA due to the route of feeding. This suggested that the appropriate ratio of BCAA might be different and specific for each route of feeding. We decided to use the IAAO method to determine whether the ratio among the BCAA used was appropriate for enteral and parenteral feeding.

The IAAO method has been successfully used in pigs and humans to determine the amino acid requirements of indispensable amino acids (Brunton et al, 1998 and Pencharz & Ball, 2003) The primary assumption of the technique is that the partitioning of any indispensable amino acid between oxidation and protein synthesis is sensitive to the most limiting amino acid provided in the diet (Zello et al, 1995). Thus, when the most limiting indispensable amino acid is supplied protein synthesis will increase and oxidation of all amino acids including the indicator amino acid will decrease (**Figure 4.5**). This concept was applied to the present experiment by providing a deficient intake of all the BCAA in a fixed ratio and then supplementing each BCAA individually. All other indispensable amino acids were provided in excess of their requirement. Therefore the BCAA that reduces the oxidation of the indicator must be the limiting amino acid.

Based on the mean total BCAA requirement for neonatal piglets, as determined previously, the piglets in the current experiment were fed at 75% of the parenteral requirement (1.1 g/ (kg · d) and 75% of the enteral requirement (2.0 g/ (kg · d) with the previous ratio of BCAA and the oxidation of the indicator amino acid, phenylalanine, was determined. The piglets were then supplied with either isoleucine,

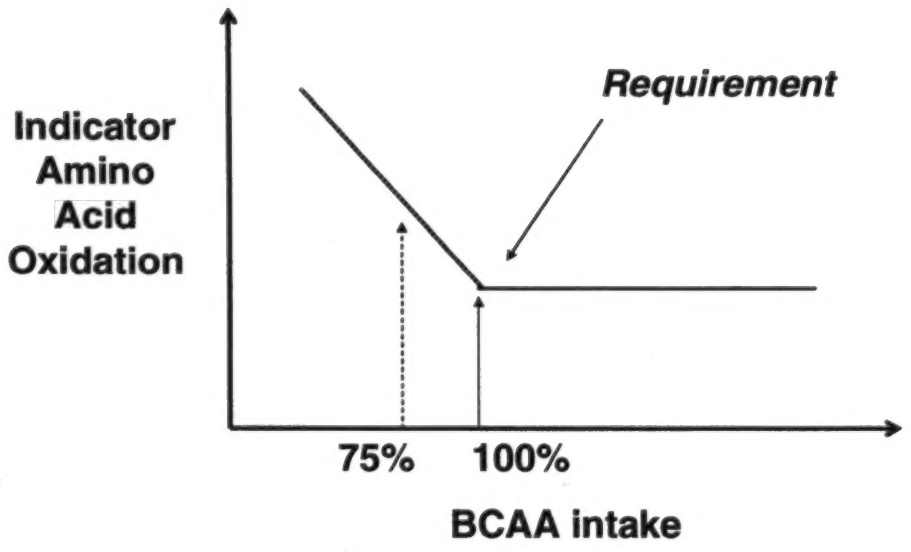

Figure 4.5 Experimental Design Concept: Oxidation of indicator amino acid in response to supplementation of the most limiting branched-chain amino acid (BCAA). Illustrated is the oxidation response of indicator amino acid [L-1-^{14}C-phenylalanine], in response to the individual supplementation of most limiting BCAA (isoleucine, leucine or valine) to 75% of parenteral [1.1g/(kg · d)] and enteral [2.0 g/(kg · d)] requirement, to meet 100% of total BCAA requirement; 100% being 1.53 and 2.64 g/(kg · d) for parenteral and enteral feeding, respectively.

leucine or valine individually to meet 100% of parenteral (1.53 g/(kg · d)) and enteral (2.64 g/(kg · d)) requirement (Table 4.1). Supplementing the most limiting BCAA would increase the uptake of amino acids for protein synthesis and thus decrease the oxidation of phenylalanine. BCAA exhibit antagonism, where addition or deletion of any one individual BCAA influences the metabolism and thus requirement of the other two BCAA (Harper et al, 1984). We chose the 25% addition level of each individual BCAA to observe a sensitive change in phenylalanine oxidation and reduce potential BCAA antagonistic effects.

In experiment 1-parenteral BCAA ratio, significant decreases ($P < 0.05$) in percentage of dose oxidized and Δ were observed in phenylalanine oxidation from 75% BCAA diets due to addition of isoleucine (12.6%) and valine (6.6%) (Figs. 1 & 3), while the supplementation of leucine had no significant effect (~2%) (Figure 4.2). Thus, the most limiting BCAA for protein synthesis during parenteral nutrition under the current ratio of 1:1.8:1.2, isoleucine/leucine/valine is isoleucine, followed by the second limiting amino acid, valine. Parenteral nutrition by passes first pass metabolism by the splanchnic organs and thus nutrients including amino acids are provided to the peripheral organs at different concentrations than via the oral route. The current ratio among BCAA is based on oral requirement studies, and the pattern of BCAA in reference proteins such as human milk (Elango et al, 2002: Chapter 3). The results from the current study indicate that during parenteral nutrition the ratio of 1:1.8:1.2 of isoleucine/leucine/valine is inappropriate.

Supplementation of individual BCAA to the parenteral test diet resulted in the expected increase in the plasma concentration of the BCAA which was added, for

isoleucine and for valine; however there was no increase in plasma leucine when the intake of parenteral leucine was increased. The addition of parenteral isoleucine to the 75% BCAA diet resulted in an almost 5 fold increase in plasma leucine, presumably due to a reduction in leucine oxidation. Further, the decrease in plasma concentration of lysine and threonine observed when isoleucine was supplemented in the diet supports the interpretation that isoleucine was first limiting.

To the authors' knowledge the current experiment is the only study examining the optimum ratio of BCAA during parenteral nutrition in pigs. Iwasawa et al (1991) examined the optimal ratio of individual BCAA in injured rats being fed parenterally. They maintained isoleucine/valine at a ratio of 1:1 and supplemented leucine at 0.5, 1, 2 and 4 on a molar ratio basis and measured nitrogen balance, urinary 3-methyl-histidine concentrations and plasma-free amino acids over a period of 7 days. They observed no changes in mean cumulative 7-day nitrogen balance and 3-methyl-histidine excretion. Infusion of BCAA solutions 1:0.5:1 and 1:4:1 (isoleucine/leucine/valine) caused significant changes in plasma BCAA concentrations from pre-infusion values and BCAA solutions of 1:1:1 and 1:2:1 (isoleucine/leucine/valine) tended to allow BCAA concentrations to approach pre-infusion values. The researchers concluded that the optimal ratio of BCAA during parenteral nutrition in the injured rat model lies between 1:1:1 and 1:2:1. Bonau et al. (1984) examined the relationship between composition and efficacy of enriched BCAA amino acid solutions in post-operative adult surgical patients receiving parenteral nutrition. 3 BCAA solutions –one with a ratio of 1:1.28:1.2, but 25% BCAA enriched and the other two at a ratio of 1:0.24:1.6 and 1:2:1.1(isoleucine/leucine/valine) at 45% BCAA

enrichment of total amino acids were tested over a period of 7 days. Whole body protein kinetic studies conducted using infusion of ^{15}N-glycine on 3 and 4 days post-operative indicated no significant changes in whole body protein flux, but protein catabolism was significantly higher in the group infused 1:0.24:1.6, followed by 1:2:1.1 group and lowest in the 1:1.28:1.2 group. In addition nitrogen balance was highest in the group infused with 1:1.28:1.2 (isoleucine/leucine/valine). As observed in the current study, both the previous mentioned studies indicate that the appropriate ratio among BCAA during parenteral nutrition is closer to 1:1:1 of isoleucine/leucine/valine.

In experiment 2-enteral BCAA ratio, phenylalanine oxidation (percentage of dose oxidized and Δ) did not significantly change due to individual addition of any of the BCAA (Figures 4.1, 4.2, 4.3 and 4.4). This demonstrates that during enteral feeding, all three BCAA were co-limiting and thus no change in indicator amino acid oxidation was observed. The ratio of 1:1.8:1.2 of isoleucine/leucine/valine used in the initial total BCAA requirement study (Elango et al, 2002: Chapter 3) is appropriate during enteral feeding in neonatal piglets. The NRC (1998) recommended BCAA ratio was predominantly derived from oral feeding studies and is thus optimal.

We recently reported a similar study in orally fed adult males in which we demonstrated that valine was the most limiting BCAA amino acid in egg protein (Riazi et al, 2003). In the current study, although the addition of valine did not reach significance ($P = 0.08$), none-the-less there was a numeric reduction in phenylalanine oxidation, which is consistent with our human studies. The pattern of BCAA used in the current piglet study is similar to human milk, rather than egg, and the proportional content of valine is higher in milk than in egg. Based on a comparison of the present study with that

of Riazi et al. (2003) it appears that the BCAA balance in milk protein is more ideal for enteral feeding than the pattern of BCAA in egg protein.

In the enterally fed piglets supplementation of leucine to the 75% BCAA diet resulted in 10 fold reduction in plasma isoleucine concentrations, but had no significant effect on valine (Table 4.3). Similarly supplementation of valine reduced plasma isoleucine concentrations by almost 3 fold. The addition of leucine and valine to the 75% enteral BCAA diet appeared to antagonize isoleucine metabolism. In orally fed adult humans, Pelletier et al (1991a & 1991b) examined BCAA interactions and the relative effect of each on amino acid requirement. In the first set of experiments (1991a), the authors examined valine metabolism at different leucine intakes, keeping isoleucine constant. And in the second set of experiments (1991b), leucine metabolism was examined at different valine and isoleucine intakes. Their results indicated that within the range of intakes studied, the interactive effect on the remaining BCAA was minimal and it did not interfere with the requirement values for leucine or valine. In the current study we examined the relative change in phenylalanine oxidation and plasma concentrations using diets formulated to supply 75%, a deficient intake of the mean requirement for total BCAA. Although there was no significant difference in indicator amino acid oxidation, meaning that the three BCAA were co-limiting for protein synthesis, the plasma concentrations of isoleucine suggested that some form of antagonism was occurring.

Phenylalanine oxidation as a percent of dose was observed to be higher in the parenterally fed piglets compared to the enterally fed pigs, although the route isotope infusion was parenteral in both groups. This suggests that during enteral feeding there is a greater partitioning of phenylalanine to protein synthesis during first pass by the

splanchnic tissues. Phenylalanine utilization by the gut and liver has also been reported by Stoll and colleagues (1998) during enteral infusion of ^{13}C algal protein. In our previous studies of threonine (Bertolo et al, 1998) and methionine (Shoveller et al, 2003) requirements we observed similar differences in phenylalanine oxidation due to route of feeding. Further, in order to determine whether the route of isotope infusion has an effect on the IAAO requirement estimates, we determined tryptophan requirements during enteral and parenteral routes of 1-^{14}C-phenylalanine infusion (Cvitkovic et al, 2004); and we found no significant influence on the requirement estimation due to route of isotope infusion.

With respect to the large differences in plasma valine and isoleucine, but not leucine concentration between the enteral and parenteral groups, the explanation probably involves the BCAA catabolizing enzymes. In addition to the general branched-chain amino transferase (BCAT) and branched-chain dehydrogenase (BCDH) complex, leucine specific BCAT and BCDH have been reported in various tissues and species (Harper et al, 1984); This is probably one of the reasons why leucine utilization rate is greater compared to valine and isoleucine. Leucine has also been reported to activate BCDH activity in rat tissues (Frick et al, 1980). We previously observed that with graded supplementation of total BCAA, plasma leucine concentrations were consistently lower (Elango et al, 2002: Chapter 3), compared to plasma isoleucine and valine concentrations. The BCAT isozyme for valine has a higher Km when compared to the isozymes for leucine and isoleucine and thus results in lower rate of clearance from the plasma pool (Staten et al, 1984). Further, Staten et al (1984) also reported that valine concentrations were high in plasma, but ketoisovalerate (KIV) concentrations were half of

ketoisocaproate (KIC) concentrations in plasma. Thus it appears that while BCDH may be the enzyme regulating BCAA decarboyxlation step, the transamination step regulated by BCAT may regulate individual plasma BCAA concentrations. Information on BCAA catabolizing enzymes is extensively available for liver and muscle tissues, but scarce for the intestinal cells. The previous study (Elango et al, 2002: Chapter 3) and the present study clearly indicate a significant influence by the gut on BCAA metabolism and further studies need to be conducted to examine the activity and regulation of BCAA enzymes in the small intestine.

In conclusion, the current study combined with the previous study (Elango et al, 2002: Chapter 3) clearly demonstrates that not only does the total BCAA requirement differ between the two routes of feeding - parenteral and enteral, the optimal ratio among the BCAA also differs. The currently used ratio of 1:1.8:1.2 (isoleucine/leucine/valine) is inappropriate during parenteral nutrition, with isoleucine being first limiting and valine second limiting for protein synthesis. We suggest that an improved ratio for parenteral feeding would be 1:1:1 (isoleucine/leucine/valine). During enteral nutrition, the ratio found in milk protein of 1:1.8:1.2 (isoleucine/leucine/valine) is close to optimal. Thus future studies in BCAA supplementation need to be conducted with the appropriate ratio of BCAA suited for each route of feeding.

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5.0 BRANCHED-CHAIN AMINO ACID CATABOLISM OCCURS IN THE PORCINE SMALL INTESTINE

5.1 Introduction

The total branched-chain amino acid (BCAA) requirements during parenteral and enteral routes of feeding was determined in neonatal piglets (Elango et al, 2002: Chapter 3). The total BCAA enteral requirements were ~44% higher than the parenteral requirements. This suggested that the small intestine was utilizing a considerable amount of the enterally delivered BCAA. This agreed with data from portal mass balance (Stoll et al, 1998) showing that approximately 40% of BCAA were utilized by the portal drained viscera. The ratio among the BCAA between the two routes of feeding was then examined to determine whether any of the BCAA were more or less utilized during parenteral or enteral feeding (Elango et al, 2004: Chapter 4). When the ratio among the BCAA in the dietary supply was altered, significant differences in plasma concentrations of BCAA and indicator oxidation were observed due to the route of feeding (Elango et al, 2004: Chapter 4). The optimal enteral BCAA ratio was determined to be 1:1.8:1.2 (isoleucine/leucine/valine) and the parenteral BCAA ratio was suggested to be 1:1:1 (isoleucine/leucine/valine). These data showed that the small intestine preferentially utilizes leucine relative to the other BCAA. The reasons for the small intestinal utilization of the BCAA are not clear. BCAA utilization could include: partial or complete catabolism, conversion to other metabolites, uptake for protein synthesis, or microbial utilization. An in-vitro study of BCAA enzymes and catabolism in pig enterocytes should resolve some of these questions.

BCAA, unlike other amino acids are catabolized by a variety of tissues having a wide range of catabolic potential (Harper et al, 1984). The reason for this is due to the presence of the 2 key BCAA catabolizing enzymes, branched-chain aminotransferase [BCAT; EC 2.6.1.42] and branched-chain ketoacid dehydrogenase [BCDH; EC 1.2.4.4] in many tissues at different activities (Chuang & Shih, 1995). BCAT is the first enzyme involved in the catabolism of the BCAA, and catalyzes the reversible transamination of the nitrogen group from the branched-chain amino acids. BCAT activity and subcellular distribution of the enzyme has been reported in rats (Hutson, 1988). Isoforms of the BCAT have been reported: isoenzyme I, predominantly mitochondrial, isoenzyme II, leucine-methionine specific, and isoenzyme III, predominantly cytosolic (Hutson et al, 1988 & 1992). Isoenzyme I, the mitochondrial BCAT (BCATm), is the most prevalent form in tissues of rats and humans (Lal & Chugh, 1995).

BCDH, the second and key enzyme which commits the carbon skeleton to oxidative decarboxylation is strictly mitochondrial (Harper et al, 1984). Over the past few decades, many studies have been published on the two BCAA enzymes in rats; these reported low BCATm and high BCDH in liver and high BCATm and low BCDH in skeletal muscle. This led to the conclusion that dietary BCAA escape splanchnic tissues intact to be transaminated in skeletal muscle, and the ketoacids circulated back to the liver, where the high BCDH then catabolized the ketoacids to CO_2. Recently, Suryawan et al (1998) compared BCAA catabolizing enzymes in rats and humans and clearly showed that the pattern of enzyme distribution was different between the two species. Human small intestine and liver had appreciable BCAT and BCDH activity as compared

to the rat liver, which had negligible BCAT and high BCDH activity (Suryawan et al, 1998) indicating that rats may not be an appropriate model for questions relating to BCAA metabolism in humans.

To the best of the authors' knowledge BCAT activity and BCAA catabolic potential in porcine tissues have not been reported. Specifically, no data exists on BCAA enzymes in porcine small intestine. Thus to pursue an explanation of our previous research (Elango et al, 2002 & 2004), we examined BCAT activity and BCAA oxidative capacity in porcine enterocytes and liver. Preliminary studies showed that the methods developed for rat tissues were either inappropriate or not optimal for use in porcine tissues. Therefore, our first objective was to develop and validate methods to isolate viable porcine enterocytes, and develop methods to measure BCAT activity and BCAA oxidation in porcine tissues. The second objective was to compare BCAT activity and BCAA oxidation potential in heart, liver and enterocytes of the pig.

5.2 Materials and Methods

5.2.1 ***Animals and tissue sampling*** : The Animal Care Committee of the University of Alberta approved all procedures used in this experiment. Tissue samples from slaughter pigs (~110 – 120kg), n=4, were obtained from a commercial abattoir (Edmonton Custom Packers, Edmonton, AB, Canada). The animals were killed by normal commercial procedure: application of high voltage directly to the brain and quickly exsanguinated. Jejunal sections from the small intestine were excised and stored in cold PBS/5mM DTT (pH 7.4). Sections of liver and heart were also obtained from the

same animals and stored in cold 0.3M mannitol /1mM EDTA. The samples were immediately (~45 min) transferred to the laboratory on ice for further processing.

5.2.2 ***Isolation of enterocytes*** : The enterocyte isolation protocol was modified and adapted from previously published methods (Watford et al, 1979, Masola and Evered, 1984, Wu et al, 1994 and Hansen et al, 2000). The protocol isolated primarily enterocytes but approximately 10% of the cells were a mixture of other mucosal epithelial cells including: intraepithelial lymphoid cells, goblet cells and paneth cells. Following the lead of previous publications the isolated cells will be referred to in this thesis as enterocytes. Intestinal sections were measured (~150cm) and rinsed gently with cold (4°C) PBS/5 mM DTT [PBS 137mM NaCl / 2.7mM KCl / 4.3 mM Na_2HPO_4 / 1.4 mM KH_2PO_4] to remove any residual dietary contents. The sections were rinsed gently with the incubation media [Krebs Henseleit Bicarbonate (KHB) Ca^{2+}-Free buffer]. The KHB buffer was supplemented with 10 mM Hepes, 1mM DTT, 2mM EGTA, 20mM DL-glucose, 2.1mM lactate and 0.3mM pyruvate and pH adjusted to 7.4. The KHB buffer was also pre-warmed to 37°C and oxygenated (O_2:CO_2 , 95% : 5%).

The sections were clamped at one end and filled with pre-warmed KHB buffer (~800mls/150cm intestinal section). The other side was clamped. The filled intestinal sections were incubated in a shaking water bath for 45 - 60 minutes. The sections were gently massaged at regular intervals during the incubation and the system was oxygenated continuously. This cell isolation technique removed enterocytes from along the midvillus and villus tips of the jejunum. Following the incubation, the cell suspension inside the intestinal sections were emptied into a beaker and the volume noted. The cell suspension/slurry was filtered through a layer of cheesecloth. The filtered cell

suspensions were centrifuged at 600 g for 5 minutes at room temperature. After removal of the supernatant, the pellets were gently re-suspended in the KHB buffer. The washing step was repeated three times. When mitochondria were required for enzyme assays the final enterocyte pellet was re-suspended in 250mM sucrose/5mM HEPES/5mM DTT (pH 7.4). When intact enterocytes were needed for the oxidation assays, the final re-suspension media contained KHB/0.1% BSA/2mM DTT. Cell viability assessed by cellular exclusion of 0.2% Trypan blue solution and was found to be >90%.

5.2.3 ***Isolation of mitochondria*** : The re-suspended enterocyte pellet [in 250mM sucrose/5mM HEPES/5mM DTT (pH 7.4)] was transferred to a Dounce homogenizer (Wheaton Science Products, Millville, NJ) and the cell suspension disrupted. Mitochondria from the enterocytes were obtained by differential centrifugation. The final enterocyte mitochondrial pellet was re-suspended in 0.3M mannitol/2mM DTT

Liver and heart tissues (20g) were weighed and homogenized (VirTis tissue homogenizer, VirTis, Gardiner, NY). Mitochondria were isolated from liver and heart by differential centrifugation (Miller and Harper, 1988). Mitochondrial pellets from liver and heart were re-suspended in 0.3M mannitol.

5.2.4 ***Protein determination***: Protein concentrations of mitochondria and intact enterocytes were determined by the Bicinchoninic acid (BCA) technique, (Sigma-BCA1) and diluted to the protein concentrations required for each tissue and assay, as determined by the respective protein curve.

5.2.5 ***Branched-chain aminotransferase (BCAT) assay***:A coupled enzymatic assay was used to determine the specific activity of BCAT in various porcine tissue mitochondria. The assay principle was based on a method described by

Schadewaldt (2000). α-Ketoisocaproic acid and glutamate were provided as the substrates to mitochondrial preparations and the reversible transamination reaction forming leucine and α-ketoglutarate was measured (**Figure 5.1**). The α-ketoglutarate

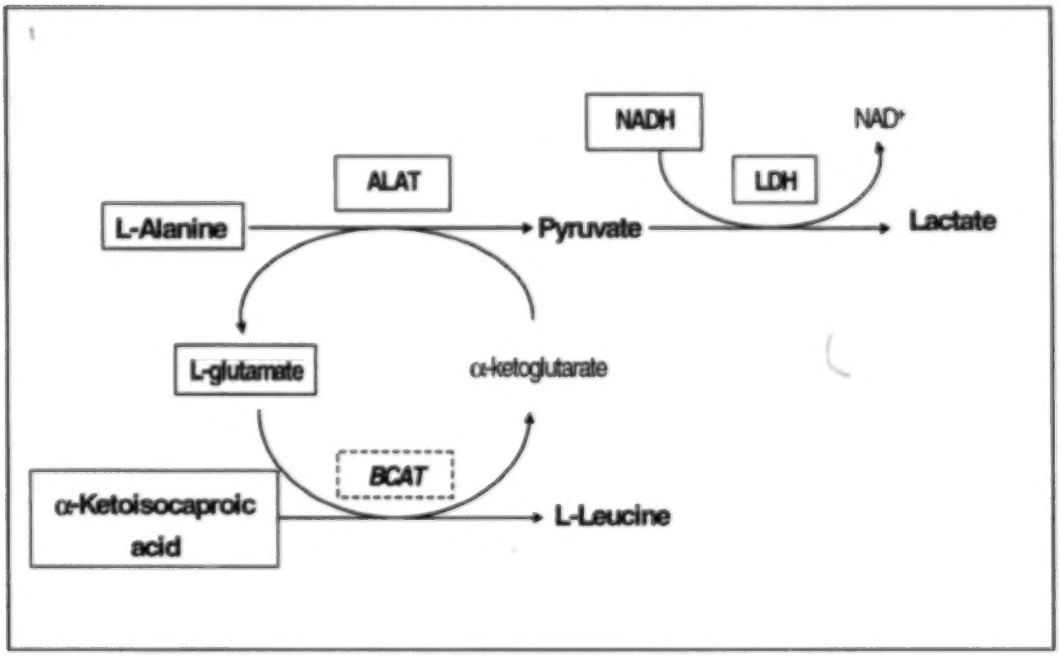

Figure 5.1 Coupled enzymatic assay to measure branched-chain aminotransferase (BCATm) activity

Principle. Substrates (within solid boxes) were added in the assay media and the reaction of BCAT (within dashed box) catalyzing Leucine formation from α-ketoisocaproic acid(KIC) was made favorable. Spectrophotometric disappearance of NADH was observed over time. Leucine appearance was confirmed by HPLC analysis.

Abbreviations. ALAT, alanine aminotransferase; BCAT, branched-chain aminotransferase; LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide hydrogen.

formed is coupled with alanine amino transferase (ALAT) plus lactate dehydrogenase (LDH) as the indicator systems. The transaminating rate was continuously monitored by the disappearance of NADH to form NAD^+, in a spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). The original assay was modified to be analyzed in a 96-well microplate which allowed simultaneous and continuous measurement of transamination in several different tissues.

The total reaction volume was 300µl per well in the microplate. Each well consisted of 70µl of Tris-Glu-Ala-NaOH buffer, pH 8.3 [0.2M Tris, 0.6M L-glutamate, 0.4M L-alanine, pH adjusted with 2M NaOH]. 20µl of 4mM NADH, 20µl of 2mM pyridoxal phosphate, 20µl of LDH, 30µl of ddH_2O, 100µl of solubilized mitochondria and 20µl of ketoisocaproic acid (KIC). The reaction was initiated with 20µl of ALAT [mixed in enzyme dissolution buffer containing 100mM potassium phosphate buffer, pH 7.4, to a final activity concentration of 10mM/min]. The disappearance of NADH was measured spectrophotometrically [absorbance of 340nm] for 15 min at 25°C. The enzyme assay was verified to be linear with time (0 – 20 min) and protein (heart 0.1 – 2.0mg, liver 0.1 – 3mg and enterocyte 0.5 – 5mg).

5.2.6 ***Branched-chain amino acid oxidation*** : Intact enterocytes, and liver mitochondria were used for the oxidation assays. The assay was based on Miller and Harper (1988) and Hutson (1986) and were performed in duplicates. The assay media contained 148 mM mannitol, 48mM Sucrose, 79mM HEPES, 25mM KH_2PO_4, 2mM $MgCl_2$, 1mM EGTA, 8mM Na_2CO_3, 1.9mM NAD, 1.0mM Coenzyme A, 0.2mM TPP, 3mM α-KG, 0.05mM malate (sodium salt). The oxidation was performed in 25ml Erlenmeyer flasks with 2ml of the oxidation media which contained any one of the three

BCAA (1-^{14}C-labelled isoleucine, leucine or valine) or 1-^{14}C-KIC. 1ml of intact enterocyte or mitochondrial liver protein, which ranged between 10-20 mg protein/ml, was added to start the reaction. The flasks were capped with a rubber stopper which had a center well containing 300µL of CO_2 absorber (ethylene glycol monomethyl ether: ethanolamine, 2:1, v/v). The assay was performed for 3 hrs at 30°C in a shaking water bath, and the reaction was stopped by injecting 1ml of 0.6M sodium citrate (pH 3.0). The flasks were allowed to continue shaking in the water bath for an additional 60 min to ensure complete collection and trapping of CO_2. The center wells were transferred to scintillation vials, mixed with 5ml of Atomlight (Packard BioScience B.V., Groningen, The Netherlands) and the radioactivity counted in a liquid scintillation counter (Beckman LS 5801, Beckman Coulter Canada Inc., Mississauga, ON). The oxidation assay was verified for linearity with protein (0 – 40mg) and time (0 – 3hr) for the oxidation of leucine in both intact enterocytes and liver mitochondria.

5.2.7 ***Inhibition assays for leucine oxidation***: Potassium fluoride has been shown to inhibit phosphatases and thus prevent BCDH activation (Aftring et al, 1986). Thus leucine oxidation was measured at 0, 10, 50 and 100mM potassium fluoride in enterocyte and liver assay preparations to verify that the oxidation assay procedure was being regulated by BCDH.

5.2.8 ***Leucine oxidation in different enterocyte preparations*** : The enterocyte isolation procedure yielded an appreciable number of viable intact enterocytes. However, mitochondrial protein yield from the enterocytes was not sufficient to perform all necessary oxidation experiments; thus intact enterocytes were used for the BCAA oxidation assays. In order to compare the differences in oxidation rates among the

enterocyte preparations we conducted leucine oxidation studies in intact enterocytes, enterocyte homogenate and enterocyte mitochondria.

5.2.9 ***Calculations*** :

BCATm activity was calculated as

$$\text{Specific Activity} = \frac{\text{Total assay volume}}{\varepsilon \text{ NADH x Pathlength x volume used x [protein]}} \times \frac{\text{Absorbance}}{\text{Time}}$$

Where,

ε NADH = 6.22 /mM and pathlength = 0.638 cm

5.2.10 ***Chemicals*** : L-[1-^{14}C]-isoleucine (specific activity 54mCi/mmol) and L-[1-^{14}C]-valine (specific activity 55mCi/mmol) were purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO. L-[1-^{14}C]-leucine (Specific activity 58mCi/mmol) and L-[1-^{14}C]-ketoisocaproic acid (specific activity 56mCi/mmol) were purchased from Amersham Biosciences Corp., Piscataway, NJ. All other laboratory chemicals were purchased from Sigma (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) unless specified otherwise.

5.2.11 ***Statistical analyses*** : Data are presented as means ± SEM. One-way ANOVA (SAS/STAT version 8.01, SAS institute, Cary, NC) was performed, employing PROC GLM procedure with the specific activity of BCAT, BCAA oxidation or KIC oxidation as the main effect. Differences between tissues for BCAT specific activity and differences among BCAA and KIC oxidation rates were assessed by least square means procedure. Data were considered significant at $P < 0.05$.

5.3 Results

5.3.1 ***Branched-chain aminotransferase activity***: BCATm activity was significantly ($P < 0.05$) different among heart, enterocyte and liver (**Table 5.1**). Although enterocyte specific activity of BCATm was the lowest, it was 20% of the activity found in heart.

5.3.2 ***Branched-chain amino acid oxidation in intact enterocytes*** : Intact enterocytes of pigs are capable of oxidizing isoleucine, valine, leucine and KIC to CO_2 (**Figure 5.2**). Compared to the oxidation rate of leucine (4.3 ± 2.2 nmol/mg/hr), oxidation of isoleucine (0.4 ± 0.2 nmol/mg/hr) and valine (0.6 ± 0.2 nmol/mg/hr) were significantly ($P < 0.05$) lower. The oxidation rate of KIC (3.9 ± 1.7 nmol/mg/hr) by intact enterocytes was not different from that of leucine.

5.3.3 ***Branched-chain amino acid oxidation in liver mitochondria***: The oxidation of leucine(17.9 ± 4.8 nmol/mg/hr), isoleucine (4.6 ± 2.3 nmol/mg/hr) and valine (5.9 ± 2.4 nmol/mg/hr) were not significantly different ($P > 0.05$) in liver mitochondria (**Figure 5.3**). KIC oxidation rate (43.9 ± 14.2 nmol/mg/hr) was significantly ($P < 0.05$) higher than the oxidation rate of any of the 3 BCAA.

5.3.4 ***Inhibition of leucine oxidation***: Potassium fluoride, at all concentrations tested, inhibited leucine oxidation significantly ($P < 0.05$) in both enterocyte and liver mitochondria (**Figure 5.4**). At 10mM potassium fluoride, oxidation was 12% of the control oxidation rate in enterocytes. There was a small, but significant increase in inhibition (6.3% of control) at 100mM in enterocytes.

Table 5.1 Branched-chain aminotransferase (BCATm) activity in adult porcine tissues[1]

Tissue	Specific Activity[2] (μmol/min/mg protein)	% of Heart
Heart	2.55 ± 0.11^{a}	100
Enterocyte	0.51 ± 0.01^{b}	20.0
Liver	1.52 ± 0.51^{c}	59.6

Values are means ± SEM; n=5

[1] Disrupted mitochondrial preparations used for all tissues

[2] Values with different superscript letters are significantly ($P < 0.05$) different

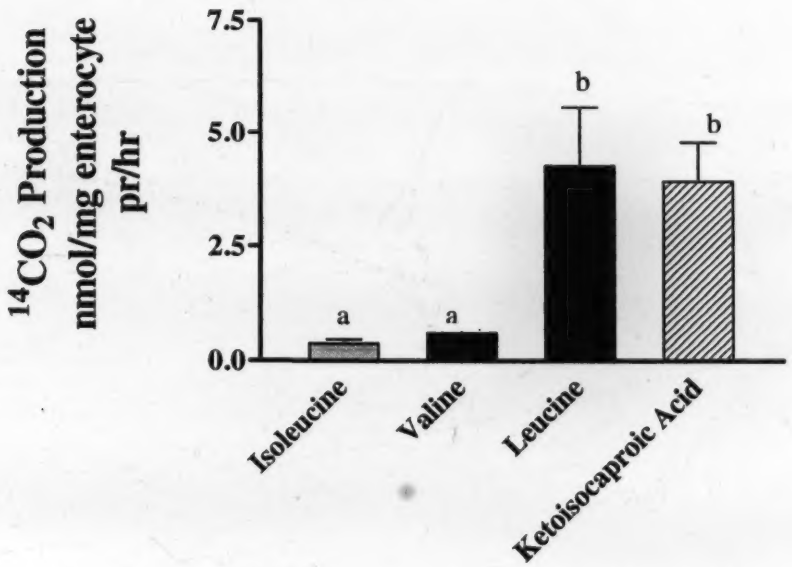

Figure 5.2 Branched-chain amino acid oxidation (nmol/mg pr/hr) in porcine intact enterocytes

Values are means ± SEM; n=4; Bars with different superscript letters are significantly ($P < 0.05$) different

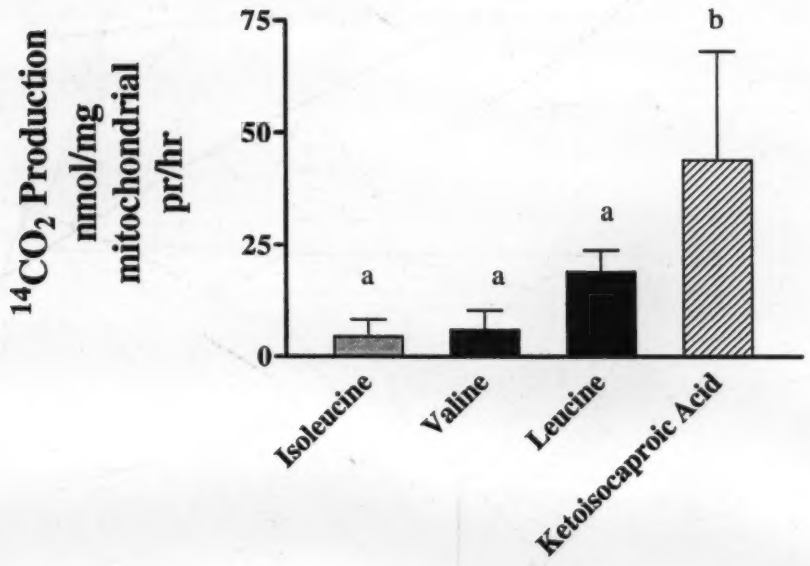

Figure 5.3: Branched-chain amino acid oxidation (nmol/mg pr/hr) in porcine liver mitochondria

Values are means ± SEM; n=4; Bars with different superscript letters are significantly ($P < 0.05$) different

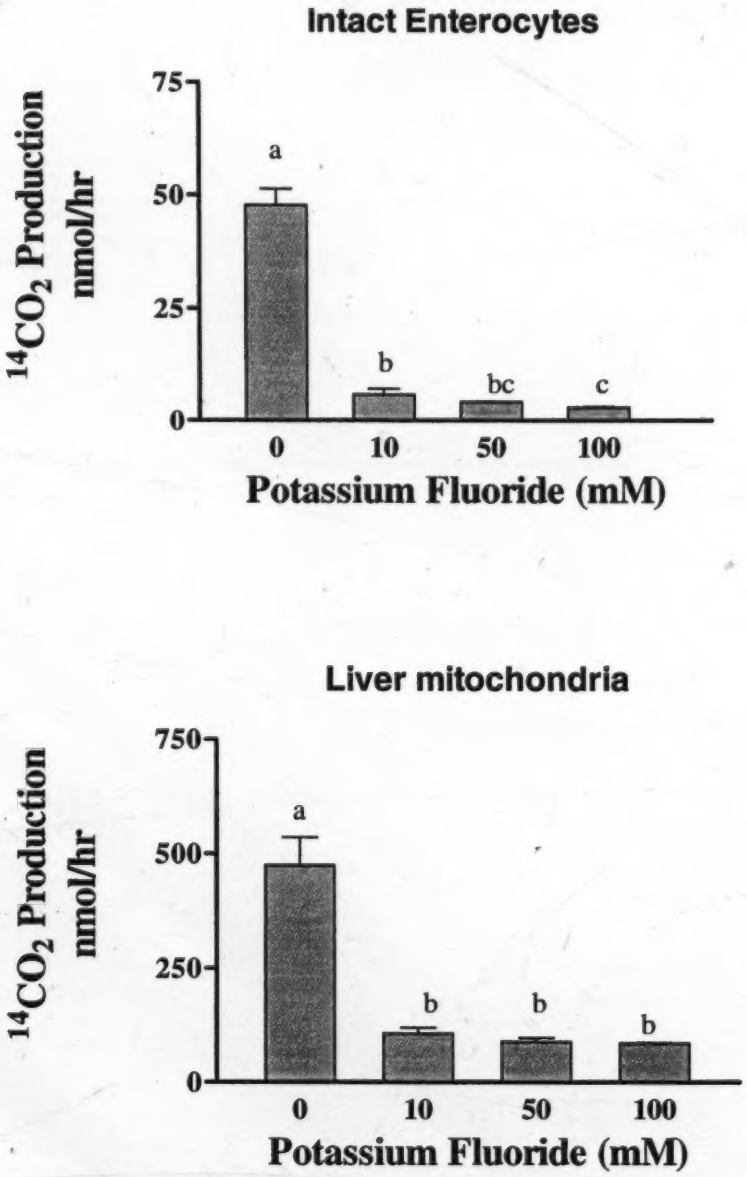

Figure 5.4 Potassium fluoride inhibition assays for leucine oxidation (nmol/hr) in enterocytes and liver mitochondria

Values are means ± SEM; n=4; Bars with different superscript letters are significantly ($P < 0.05$) different

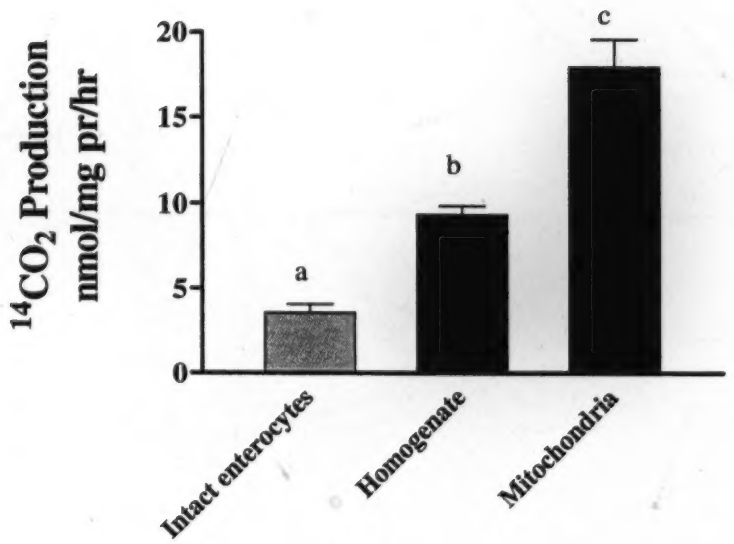

Figure 5.5 Leucine oxidation (nmol/mg pr/hr) in intact enterocytes, enterocyte homogenate and enterocyte mitochondrial preparations

Values are means ± SEM; n=4; Bars with different superscript letters are significantly ($P < 0.05$) different

5.3.5 ***Leucine oxidation in different enterocyte preparations***: There were significant differences in leucine oxidation among the 3 enterocyte preparations (**Figure 5.5**). Enterocyte mitochondria had the highest oxidative rate (18.0 ± 1.63 nmol/mg/hr), which was significantly higher ($P < 0.05$) than the intact enterocyte preparation (3.6 ± 0.5 nmol/mg/hr) and the homogenate preparation (9.4 ± 0.5 nmol/mg/hr), which were different from each other.

5.4 Discussion

In spite of reports of involvement of the splanchnic tissues in essential amino acid metabolism (Gelfand et al, 1986), specific interest in small intestinal metabolism of essential amino acids came about only recently (Stoll et al, 1998 & Bertolo et al, 1998). Stoll et al (1998) using ^{13}C- labeled tracers and portal mass balance methods in 28 d old pigs reported a portal drained visceral uptake of 43%, 39% and 31% of leucine, valine and isoleucine, respectively. We subsequently determined the total BCAA requirement in parenterally fed neonatal piglets to be ~56% of the enteral requirement, thus suggesting that ~44% of enterally delivered total BCAA was utilized by the small intestine on first pass metabolism (Elango et al, 2002: Chapter 3). Confirming biochemical evidence whether the enterocytes could catabolize the BCAA to CO_2 could not be found in the literature. Based on the previous indirect evidence (Stoll et al, 1998 and Elango et al, 2002: Chapter 3) we hypothesized that the pig enterocytes would contain BCAT and could catabolize the individual BCAA to CO_2.

Branched-chain aminotransferase specific activity was observed to be highest in pig heart, lower in liver (60% of heart) and lowest (20% of heart) in enterocyte

mitochondria (Table 5.1). In rats, heart, pancreas and stomach have the highest specific activity of BCAT, followed by adipose tissue and kidney (Torres et al, 1998). BCAT activity has been reported to be moderate in rat skeletal muscle and low in rat liver (Harper et al, 1984) and rat small intestinal mucosa (Suryawan et al, 1998). In the present study, a similar pattern of BCAT activity was observed in pig tissues, with heart mitochondria exhibiting significantly higher BCAT specific activity than liver or enterocyte mitochondria. However, BCAT is not the rate limiting enzyme for BCAA flux through the catabolic pathway in rats. BCDH, the key oxidative decarboxylating enzyme, commits the carbon skeleton of the BCAA to the oxidative pathway (Harper et al, 1984). In contrast, Matthews et al (1981) and Staten et al (1984), reported in humans that at least at the plasma concentrations level, BCAT might play a role in maintaining BCAA plasma concentrations. The general BCAT isoform for the 3 BCAA has a varied effect on the individual BCAA due to the differences in relative Km's. The BCAT isozyme for valine has a higher Km when compared to the isozymes for leucine and isoleucine and thus results in lower rate of clearance from the plasma pool (Staten et al, 1984). Further, it has also been reported that valine concentrations were high in plasma, but ketoisovalerate (KIV) concentrations were half of ketoisocaproate (KIC) concentrations in plasma (Staten et al, 1984). Thus it appears that while BCDH may be the enzyme catalyzing the committed BCAA decarboyxlation step, the transamination step catalyzed by BCAT may regulate individual plasma BCAA concentrations in humans. In pigs, it appears that the BCAT activity does not play a significant role, because in spite of the low specific activity of BCAT in both liver and enterocytes, BCAA oxidation occurred at an appreciable rate.

BCAA in-vitro oxidation studies were conducted to determine whether complete catabolism of BCAA to CO_2 occured in the small intestine. Isoleucine, valine and leucine oxidative rates were compared to KIC oxidative rates to determine whether BCAT might limit the decarboxylation step. Leucine and KIC oxidation were found to be significantly higher in enterocytes (Figure 5.2) and liver (Figure 5.3). These results have significant implications, because this is the first direct evidence that the enterocytes oxidize BCAA, specifically leucine, at appreciable rates. Recently, Burrin et al (2003) reported BCAT and BCDH activity in 28 d old piglet small intestine. They reported ~140 mU BCAT activity/min/g tissue and ~5.3 mU BCDH activity/min/g tissue in enterally fed piglet small intestine. This suggests that a portion of the dietary BCAA utilized by the intestine are unavailable to the rest of the body, due to catabolism on intestinal first pass and conversely, the BCAA, especially leucine and KIC, could act as significant energy sources to the intestinal epithelium. Windmueller (1982) first established in a series of elegant experiments that glutamine, glutamate and aspartate were significant oxidative fuels for the small intestine. BCAA are predominantly known for nitrogen transfer via alanine and asparagine, sources for protein synthesis or energy sources via oxidation (Harper et al, 1984). Based on the previous in-vivo studies (Elango et al, 2002: Chapter 3 & 2004: Chapter 4), and the current study establishing the capacity for enterocytes to oxidize BCAA to CO_2, it appears that BCAA could be an additional significant fuel for the small intestinal epithelium. BCAA concentration in most foods and thus dietary intake, are generally in excess relative to dietary requirements in pigs (NRC) and humans (Harper et al, 1984). Therefore it may be reasonable to assume that intestinal utilization of BCAA could include catabolism. This would provide energy for many uses and spare

the need to catabolize more limiting nutrients. In addition, the nitrogen released could be used for synthesis of key nitrogen containing metabolites.

KIC oxidation is expected to be higher than leucine oxidation for two reasons. First, the deamination step is not necessary for catabolism of the ketoacid. Secondly, KIC specific transporters have been reported in rat heart mitochondrial membrane (Hutson and Hall, 1993). Similar transporters existing in porcine liver mitochondria are probably the reason for higher oxidative rates for KIC, compared to BCAA, in liver mitochondria (Figure 5.3). In enterocytes, KIC and leucine had similar oxidative rates (Figure 5.2). The explanation probably involves the use of intact enterocytes versus isolated mitochondria. KIC transporters may not be present on the enterocyte membrane. This is supported by the differences in oxidation rates of leucine observed in different enterocyte preparations (Figure 5.5). Enterocyte mitochondrial oxidation of leucine was 5 fold higher than intact enterocyte oxidation. Intact enterocytes were used in the current study for comparing all the 3 BCAA and KIC oxidative rates (Figure 5.2), because of experimental constrictions with respect to total protein yield in mitochondrial fractions.

Harper and Benjamin (1984) reported an in-vivo study in rats, comparing the relationship between the intake and rate of oxidation of leucine and KIC. Dietary leucine was mixed with 1-^{14}C-leucine, and oxidation compared to dietary KIC mixed with 1-^{14}C-KIC. Whole body oxidation rates of KIC were considerably higher than leucine oxidation. These in-vivo data suggest that reamination of KIC to leucine is probably limited, and that KIC is preferentially channeled towards the oxidative pathway. The in-vitro evidence in the present study for enterocyte catabolism of BCAA and KIC,

provides the direct experimental proof that during first pass metabolism the small intestine can irreversibly catabolize BCAA to CO_2.

Fatania et al (1983) reported inhibition of BCDH activation by 50mM potassium fluoride in ox kidney. Aftring et al (1986) later reported the same in rat skeletal muscle. The BCDH complex is regulated by phosphorylation (inactive form) and dephosphorylation (active form) (Chuang and Shih, 1995). The phosphorylation reaction is catalyzed by BCDH kinase, and the dephosphorylation by BCDH phosphatase. Potassium fluoride inhibits the BCDH phosphatase and thereby makes the BCDH complex inactive. In pig enterocytes and liver mitochondria, potassium fluoride inhibited BCDH activation at 10, 50 and 100mM (Figure 5.4). This confirms that the oxidation assay of measuring BCAA 1- carbon being converted to CO_2 via the BCDH catabolic pathway is a reliable method.

In recent in-vivo studies, Metges (2000) reported that there was a significant contribution of microbial lysine, synthesized in the lumen, to the host lysine homeostasis in both pigs and humans, and this might affect tracer derived requirement estimates. In the current study, the enterocyte preparations were always in the presence of 2 – 5mM DTT, and repeated washes were performed before the final suspension of enterocytes. Thus bacterial contamination was eliminated and therefore the data presented for BCAA oxidation are a direct evidence for the pig small intestinal capability to oxidize essential amino acids.

Suryawan et al (1998), compared BCAA catabolism in rats, humans and primates, and showed that in general, the BCAT activity in rat tissues were 2-10 fold higher than human or primate tissues (Chapter 1 – Table 1.1). Also, the activity of BCDH

in rat liver was ~10 fold higher than humans or primates (Chapter 1 – Table 1.3). These huge differences between species may have resulted in misinterpretation of BCAA catabolism in humans and other species. Rat liver, due to its low BCAT and high BCDH activity was thought to allow dietary BCAA to pass through intact for use by the skeletal muscle, with the deaminated excess returned to the liver for oxidation. Both the enzyme and metabolic data for the rat clearly support this conclusion. However the work of Suryawan et al (1998), in humans, and the data presented in the present paper support the conclusion that pigs are more similar to humans, with respect to BCAA catabolizing enzymes, than the rat. We suggest that the currently accepted model of inter-organ regulation of BCAA metabolism that was developed from the rat model needs to be reevaluated in humans and pigs.

In conclusion, the methods to isolate enterocytes in pigs, and conduct in-vitro BCAA enzyme and oxidation studies in pig tissues have been established. These methods could now be employed to examine the activity and regulation of BCAA enzymes and oxidation in pigs under various conditions: during conditions of stress, such as newborn pigs when their diets are switched from an intravenous route inutero to oral feeding immediately post-partum, weaning, and gut stress. BCAA metabolism has been reported to be regulated by hormones (insulin), conditions of starvation, exercise to conserve BCAA for protein synthesis or act as energy sources (Shimomura et al, 2001). The current study showing catabolism of leucine and KIC by the enterocytes, is crucial because they could act as significant energy sources in the mammalian small intestine, and needs to be examined under varying conditions of growth and development.

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6.0 BRANCHED-CHAIN AMINO ACID CATABOLISM DURING THE EARLY STAGES OF POSTNATAL GROWTH IN THE NEONATAL PIGLET

6.1 Introduction

The small intestine undergoes numerous critical changes following two major events: birth and weaning. Birth brings about a change in route of nutrient delivery from a predominantly intravenous route (via the umbilical vein) to complete dependence on the intestine to digest and absorb nutrients (Henning 1986). The first 10 days of postnatal life in a pig involves an 80% increase in length and 30% increase in diameter of the small intestine (Smith and Jarvis, 1978). In normal suckled piglets, during the first day of life, the small intestine weight increased by 61% and length by 23% (Widdowson et al, 1976). The cellular population in the small intestinal mucosa increases during the first 3 days of life in a pig between 84% and 154% (Xu et al, 1992). The protein synthetic rate is the highest in the small intestine at this stage, when compared against the rest of the tissues. Van Der Muelen and Jansman (1997) reported in 44kg pigs, that the fractional protein synthesis (FSR) of the small intestine was the highest (37.5%/day), compared to liver (19.7%) and skeletal muscle (3.9%).

Absorption of nutrients from swallowed amniotic fluid prepares and adapts the small intestine and fetus for the abrupt postnatal transition in route of nutrient delivery (Pitkin and Reynolds, 1975 and Sangild et al, 2002). Amino acid transporters have been reported to be present in the fetal small intestine at ~90 days of gestation in pigs (Buddington and Malo, 1996) and during the first 7 days of life, various ontogenic changes in amino acid absorption specific to each amino acid have been reported

(Buddington et al, 2001 and Smith, 1988). The absorbed amino acids are not only involved in active protein synthesis, but have also been shown to act as significant respiratory fuels, especially glutamine (Windmueller, 1982) and glutamate (Reeds et al, 1996 and Wu 1998).

In previous experiments, we have shown that, the branched-chain amino acid (BCAA) requirements in neonatal piglets fed enterally was significantly higher than in piglets fed parenterally, suggesting possible utilization of BCAA by the small intestine (Elango et al, 2002: Chapter 3). We subsequently showed that porcine enterocytes from 120kg pigs can oxidize BCAA, especially leucine, to CO_2 under in-vitro conditions (Chapter 5 – Figure 5.2). Whether neonatal porcine enterocytes can oxidize BCAA to CO_2 is not known.

BCAA catabolism is influenced by dietary protein and calorie content, hormones, exercise, and physiological states, such as starvation (Shimomura et al, 2001). Catabolism of BCAA during the above mentioned conditions are increased to provide energy, or decreased to conserve amino acids for protein synthesis, especially in muscle. Whether BCAA catabolism is influenced by or is involved in the complex metabolic adaptations which take place following birth is not known. Furthermore, BCAA catabolism, unlike other essential amino acids occurs in several different tissues at various rates in mammals. Whether BCAA catabolism occurs in various neonatal piglet tissues is not yet known. Thus, in the current experiment our objectives were to determine whether neonatal enterocytes can oxidize BCAA, and also examine developmental changes in BCAA metabolism in different organs during the early postnatal stages of growth.

6.2 Materials and Methods

6.2.1 ***Animals and tissue sampling***: The Animal Care Committee of the University of Alberta approved all procedures used in this experiment. A total of 30 male Yorkshire piglets (n=15, enzyme studies; n=15, oxidation studies); 5 per age group, at 0, 3 and 7 were obtained from the Swine Research and Technology Centre, University of Alberta. All piglets were weighed, and injected with ketamine (30mg/kg) intramuscularly prior to a lethal intravenous injection of sodium pentobarbital (0.3ml/kg). Jejunal sections from the small intestine were excised and stored in cold PBS/5mM DTT (pH 7.4). Sections of liver, kidney, skeletal muscle (longismus dorsi), heart, and brain were also obtained from the same animals and stored in cold 0.3M mannitol /1mM EDTA. The samples were immediately (~45 min) transferred to the laboratory on ice for further processing.

6.2.2 ***Isolation of enterocytes*** : The enterocyte isolation protocol was modified and adapted from previously published methods (Watford et al, 1979, Masola and Evered, 1984, Wu et al, 1994 and Hansen et al, 2000). Intestinal sections were measured (~150cm) and rinsed gently with cold (4°C) PBS/5 mM DTT [PBS 137mM NaCl / 2.7mM KCl / 4.3 mM Na_2HPO_4 / 1.4 mM KH_2PO_4] to remove any residual dietary contents. The sections were rinsed gently with the incubation media [Krebs Henseleit Bicarbonate (KHB) Ca^{2+}-Free buffer]. The KHB buffer was supplemented with 10 mM Hepes, 1mM DTT, 2mM EGTA, 20mM DL-glucose, 2.1mM lactate and 0.3mM pyruvate and pH adjusted to 7.4. The KHB buffer was also pre-warmed to 37°C and oxygenated (O_2:CO_2 , 95% : 5%).

The sections were clamped at one end and filled with pre-warmed KHB buffer (~800mls/150cm intestinal section). The other side was clamped. The filled intestinal sections were incubated in a shaking water bath for 45 - 60 minutes. The sections were gently massaged at regular intervals during the incubation and the system was oxygenated continuously. This cell isolation technique removed enterocytes from along the midvillus and villus tips of the jejunum. Following the incubation, the cell suspension inside the intestinal sections were emptied into a beaker and the volume noted. The cell suspension/slurry was filtered through a layer of cheesecloth. The filtered cell suspensions were centrifuged at 600 g for 5 minutes at room temperature. After removal of the supernatant, the pellets were gently re-suspended in the KHB buffer. The washing step was repeated three times to remove all other cell debris. When mitochondria were required for enzyme assays the final enterocyte pellet was re-suspended in 250mM sucrose/5mM HEPES/5mM DTT (pH 7.4). When intact enterocytes were needed for the oxidation assays, the final re-suspension media contained KHB/0.1% BSA/2mM DTT. Cell viability assessed by cellular exclusion of 0.2% Trypan blue solution and was found to be >90%. Pictures of enterocytes were obtained through a Leitz Dialux 20 microscope (Wetzlar, Germany) by Meta Imaging Series 5.07 (Downingtown, PA) software.

6.2.3 ***Isolation of mitochondria***: The re-suspended enterocyte pellet [in 250mM sucrose/5mM HEPES/5mM DTT (pH 7.4)] was transferred to a Dounce homogenizer (Wheaton Science Products, Millville, NJ) and the cell suspension disrupted. Mitochondria from the enterocytes were obtained by differential centrifugation. The final enterocyte mitochondrial pellet was re-suspended in 0.3M mannitol/2mM DTT.

Liver, kidney, muscle, heart and brain tissues (20g) were weighed and homogenized (VirTis tissue homogenizer, VirTis, Gardiner, NY). Mitochondria were isolated from liver, kidney, muscle and heart by differential centrifugation (Miller and Harper, 1988). Mitochondrial pellets were re-suspended in 0.3M mannitol.

6.2.4 ***Protein determination***: Protein concentrations of mitochondria and intact enterocytes were determined by the Bicinchoninic acid (BCA) technique, (Sigma-BCA1) and diluted to the protein concentrations required for each tissue and assay, as determined by the respective protein curve.

6.2.5 ***Branched-chain aminotransferase (BCAT) assay*** : A coupled enzymatic assay was used to determine the specific activity of BCAT in various porcine tissue mitochondria (Schadewaldt 2000). The original assay was modified to be analyzed in a 96-well microplate which allowed simultaneous and continuous measurement of transamination in several different tissues. The total reaction volume was 300µl per well in the microplate. Each well consisted of 70µl of Tris-Glu-Ala-NaOH buffer, pH 8.3 [0.2M Tris, 0.6M L-glutamate, 0.4M L-alanine, pH adjusted with 2M NaOH]. 20µl of 4mM NADH, 20µl of 2mM pyridoxal phosphate, 20µl of LDH, 30µl of doublydeionisedH_2O, 100µl of solubilized mitochondria and 20µl of ketoisocaproic acid (KIC). The reaction was initiated with 20µl of ALAT [mixed in enzyme dissolution buffer containing 100mM potassium phosphate buffer, pH 7.4, to a final activity concentration of 10mM/min]. The transaminating rate was continuously monitored by the disappearance of NADH to form NAD^+, in a spectrophotometer (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA) [absorbance of 340nm] for 15 min at 25°C.

The enzyme assay was verified to be linear with time (0 – 20 min) and protein concentration (heart 0.1 – 2.0mg, liver 0.1 – 3mg and enterocyte 0.5 – 5mg).

6.2.6 ***Branched-chain amino acid oxidation***: Intact enterocytes, liver and muscle mitochondria were used for the oxidation assays. The assay was based on Miller and Harper (1988) and Hutson (1986) and were performed in duplicates. The assay media contained 148 mM mannitol, 48mM Sucrose, 79mM HEPES, 25mM KH_2PO_4, 2mM $MgCl_2$, 1mM EGTA, 8mM Na_2CO_3, 1.9mM NAD, 1.0mM Coenzyme A, 0.2mM TPP, 3mM α-KG, 0.05mM malate (sodium salt). The oxidation measurement was performed in 25ml Erlenmeyer flasks with 2ml of the oxidation media which contained any one of the three BCAA (1-^{14}C-labelled isoleucine, leucine or valine) or 1-^{14}C-KIC. 1ml of intact enterocyte or mitochondrial liver protein, which ranged between 10-20 mg protein/ml; was added to start the reaction. The flasks were capped with a rubber stopper which had a center well containing 300μL of CO_2 absorber (ethylene glycol monomethyl ether: ethanolamine, 2:1, v/v). The assay was performed for 3 hrs at 30°C in a shaking water bath, and the reaction was stopped by injecting 1ml of 0.6M sodium citrate (pH 3.0). The flasks were allowed to continue shaking in the water bath for an additional 60 min to ensure complete collection and trapping of CO_2. The center wells were transferred to scintillation vials, mixed with 5ml of Atomlight (Packard BioScience B.V., Groningen, The Netherlands) and the radioactivity counted in a liquid scintillation counter (Beckman LS 5801, Beckman Coulter Canada Inc., Mississauga, ON).

6.2.7 ***Calculations*** :

BCATm activity was calculated as

$$\text{Specific Activity} = \frac{\text{Total assay volume}}{\varepsilon \text{ NADH x Pathlength x volume used x [protein]}} \times \frac{\text{Absorbance}}{\text{Time}}$$

Where,

ε NADH = 6.22 /mM and pathlength = 0.638 cm

6.2.8 ***Chemicals*** : L-[1-^{14}C]-isoleucine (specific activity 54mCi/mmol) and L-[1-^{14}C]-valine (specific activity 55mCi/mmol) were purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO. L-[1-^{14}C]-leucine (Specific activity 58mCi/mmol) and L-[1-^{14}C]-ketoisocaproic acid (specific activity 56mCi/mmol) were purchased from Amersham Biosciences Corp., Piscataway, NJ. All other laboratory chemicals were purchased from Sigma (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) unless specified otherwise.

6.2.9 ***Statistical analyses*** : Data are presented as means ± SEM. ANOVA (SAS/STAT version 8.01, SAS institute, Cary, NC) was performed, employing PROC MIXED procedure with litter blocked as a random effect and the specific activity of BCATm as the main effect. Oxidation data were analyzed by employing PROC GLM procedure with pig weights and organ weights as co-variates. Differences between tissues for BCATm specific activity and differences among BCAA and KIC oxidation rates were assessed by least square means procedure. Data were considered significant at $P < 0.05$.

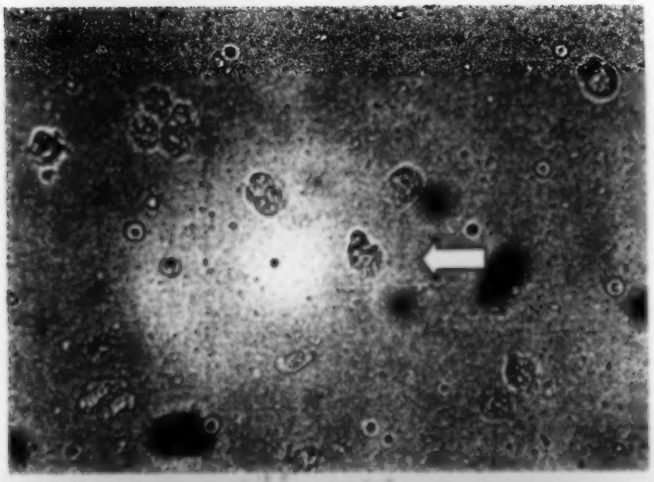
6.3 Results

6.3.1 ***Intact enterocytes*** : Isolated enterocytes showed structural maturation from birth to 7 days of age (**Figures 6.1, 6.2 and 6.3**); from a more globular form to the narrow elongated mature (columnar) enterocytes.

6.3.2 ***Branched-chain aminotransferase activity***: Significant differences ($P < 0.05$) were observed between tissues within an age group (**Table 6.1**). Skeletal muscle and heart had the highest activity, followed by intermediate activity in kidney. Liver and enterocytes had lower enzyme activity when compared to the rest of the neonatal porcine tissues (Table 6.1). As a percentage of muscle enzyme activity, enterocyte BCATm activity was 8%, 15% and 22%; liver was 10%, 18% and 10% at 0, 3 and 7 days of age respectively. Brain homogenate was used for BCAT enzyme assay, due to poor separation between mitochondrial and cytosolic fractions. No significant effect ($P > 0.05$) of age on BCATm activity was observed in tissues examined (Table 6.1).

6.3.3 ***Branched-chain amino acid oxidation in intact enterocytes***: KIC oxidation in 3 day old pigs was significantly ($P < 0.05$) lower than in 0 day and 7 day old pigs (**Figure 6.4**). No significant differences were observed between BCAA within an age group. KIC oxidation was significantly ($P < 0.05$) higher than BCAA oxidation within an age group in all 3 age groups. No significant effect ($P > 0.05$) of age was observed among BCAA oxidation in enterocytes (Figure 6.4).

6.3.4 ***Branched-chain amino acid oxidation in liver mitochondria***: KIC oxidation decreased significantly ($P < 0.05$) from 0 day old pigs to 3 and 7day old pigs



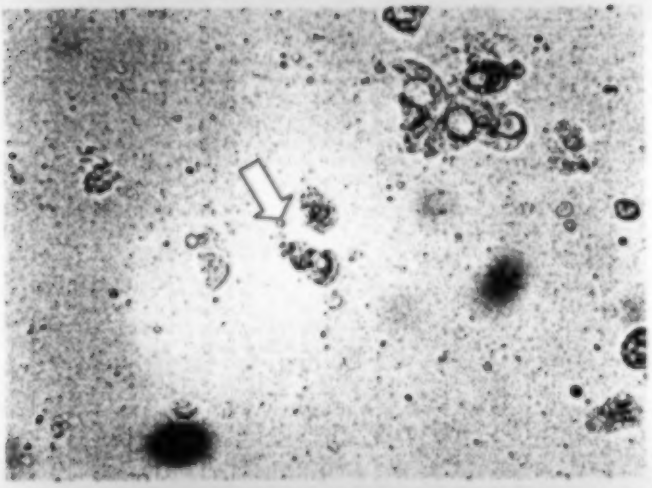
0d Pig-Enterocytes (X10)

(X25)

(X40)

Figure 6.1 Photographs of isolated intact enterocytes from 0 day old pigs

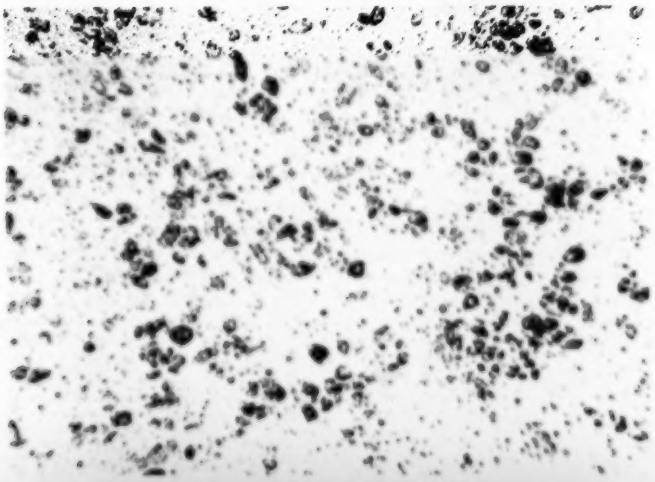
3d Pig-Enterocytes (X10)



(X25)

(X40)

Figure 6.2 Photographs of isolated intact enterocytes from 3 day old pigs



7d Pig-Enterocytes (X10)

(X25)

(X40)

Figure 6.3 Photographs of isolated intact enterocytes from 7 day old pigs

Table 6.1 Branched-chain aminotransferase (BCATm) activity in neonatal piglet tissues during early stages of postnatal growth

Tissue	0 Day Old[1]	% of muscle	3 Day Old	% of muscle	7 Day Old	% of muscle
			μmol/min/mg protein			
Enterocyte[2]	0.78 ± 0.08^{b}	8	0.95 ± 0.16^{ab}	15	2.15 ± 0.55^{a}	22
Liver[2]	1.01 ± 0.38^{b}	11	1.13 ± 0.43^{ab}	18	2.16 ± 1.19^{a}	10
Muscle[2]	9.35 ± 2.80^{a}	100	6.20 ± 1.19^{ac}	100	9.58 ± 3.61^{b}	100
Heart[2]	9.08 ± 1.63^{ac}	97	7.52 ± 2.73^{ac}	121	9.19 ± 2.20^{b}	96
Kidney[2]	1.45 ± 0.65^{bc}	16	3.82 ± 1.08^{a}	61	3.16 ± 0.92^{a}	33
Brain[3]	0.92 ± 0.65^{b}	NA	2.35 ± 0.51^{a}	NA	2.80 ± 1.13^{a}	NA

Values are means ± SEM; n=5; NA-not applicable; There were no significant differences ($P > 0.05$) among ages within a tissue; Means sharing different superscript letters within a column are different ($P < 0.05$)

[1]0 day piglets were less than 12hrs old; Litters were followed from birth through 7 days of age

[2]disrupted mitochondrial preparations were used

[3] Tissue Homogenate used for enzyme assay due to poor yield of mitochondrial versus cytosolic fractions

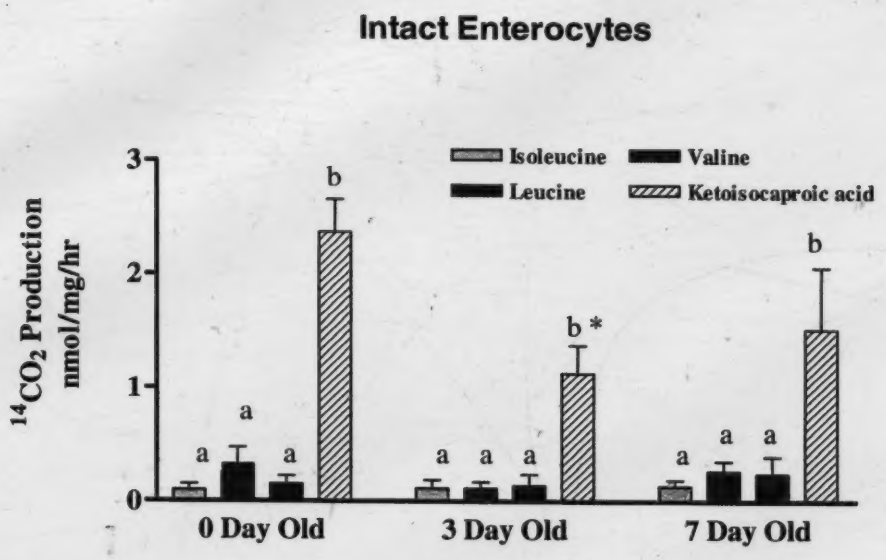


Figure 6.4 Branched-chain amino acid oxidation (nmol/mg pr/hr) in intact enterocytes during early stages of postnatal growth

Values are means ± SEM; n=5; * denotes significant ($P < 0.05$) difference between 3 day versus 0 and 7 day old pigs in KIC oxidation; Bars with different superscript letters within an age group are significantly ($P < 0.05$) different

(**Figure 6.5**). KIC oxidation was significantly higher than BCAA oxidation in 0 day and 7 day old pigs. In 3 day old pigs oxidation of leucine and KIC were significantly ($P < 0.05$) higher than isoleucine and valine. No significant ($P > 0.05$) differences among BCAA oxidation in liver mitochondria were observed with age (Figure 6.5).

6.3.5 ***Branched-chain amino acid oxidation in muscle mitochondria***: Oxidation of BCAA and KIC by muscle mitochondria steadily increased from 0 day old to 7 day old (**Figure 6.6**). Leucine oxidation increased significantly ($P < 0.05$) from 0 to 7 day old pigs. KIC oxidation increased significantly ($P < 0.05$) from 0, 3 to 7 day old pigs. Within an age group, no differences among BCAA and KIC oxidation were observed in 0 and 3 day old pigs. In 7 day old pigs, isoleucine oxidation was significantly ($P < 0.05$) lower than KIC oxidation.

6.4 Discussion

The small intestine is now recognised as an organ where the metabolic activity of the constituent cells have a considerable impact on whole-body nutrient requirements, including amino acids, during conditions of gut adaptation such as birth and weaning (McBurney 1994). In 7 day old piglets, we demonstrated that during parenteral feeding the requirement for total BCAA was 56% of the enteral requirement, suggesting that ~44% of total BCAA are utilized by the small intestine (Elango et al, 2002: Chapter 3). In subsequent experiments we showed that BCAA catabolism to CO_2 occurs in enterocytes isolated from adult pigs. However, whether neonatal enterocytes can oxidize BCAA during postnatal stages of growth when the small intestine

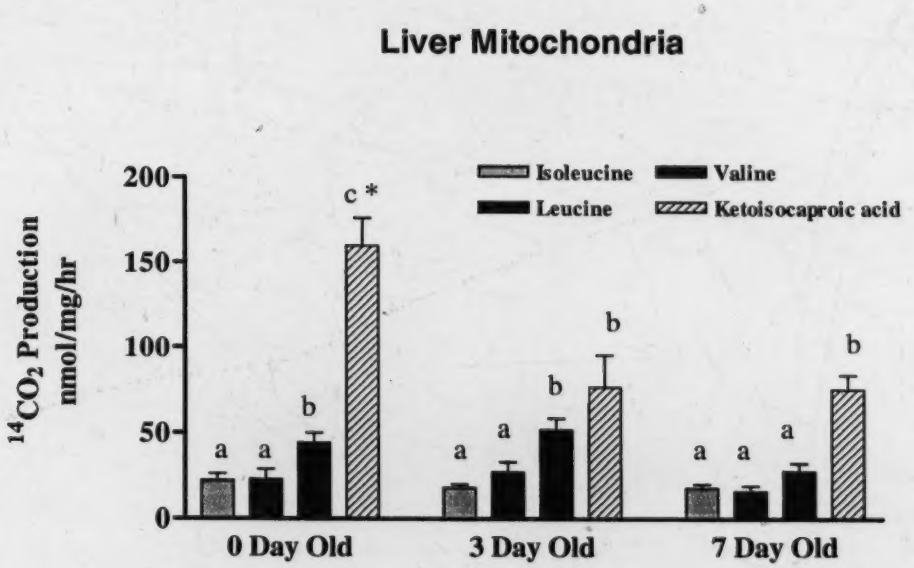


Figure 6.5 Branched-chain amino acid oxidation (nmol/mg pr/hr) in liver mitochondria during early stages of postnatal growth

Values are means ± SEM; n=5; * denotes significant ($P < 0.05$) difference between 0 day versus 3 day and 7 day old piglets in KIC oxidation; Bars with different superscript letters within an age group are significantly ($P < 0.05$) different

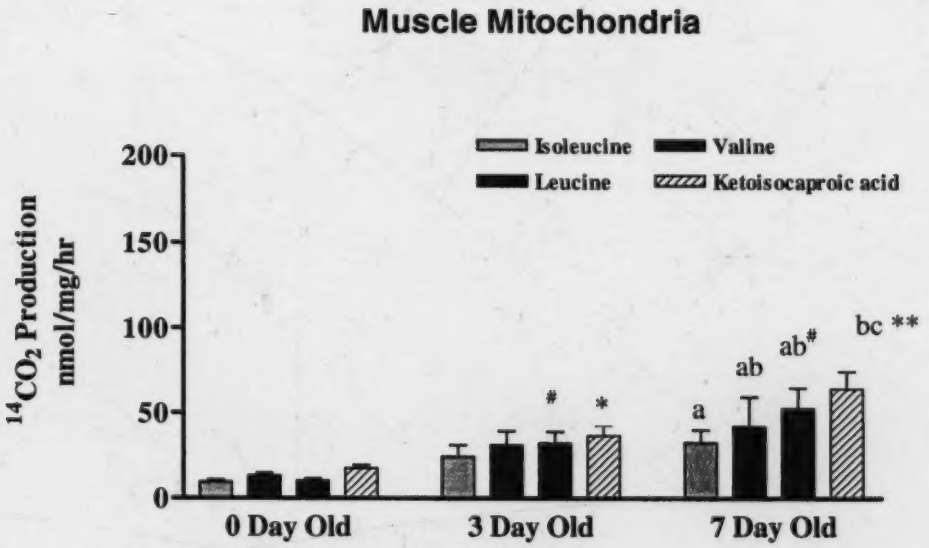


Figure 6.6 Branched-chain amino acid oxidation (nmol/mg pr/hr) in muscle mitochondria during early stages of postnatal growth

Values are means ± SEM; n=5;

* , ** denotes significant differences in KIC oxidation between 0 day versus 3 day, versus 7 day old piglets

# denotes significant differences in leucine oxidation between 0 day old versus 3 day and 7 day old piglets

Bars with different superscript letters within an age group are significantly ($P < 0.05$) different

undergoes adaptation to receive nutrients orally is not known. In the current experiment, we have demonstrated that the neonatal enterocytes can oxidize BCAA to CO_2. The second objective of this experiment was to compare BCAT enzyme activity in most piglet tissues and BCAA oxidation rates among enterocytes, liver and muscle.

BCATm activity in 7 day old neonatal enterocytes and liver mitochondria were 22% and 10% of muscle BCAT activity, respectively (Table 6.1). In rat liver and small intestine it has been reported that there is almost no BCATm activity (Hutson, 1988). Thus, it appears that in pigs, transamination of BCAA during first pass in the small intestine could be significant, and due to high BCDH activity the BCKA formed do not escape intact from the splanchnic region. Similar to rats, pig heart and muscle showed the highest BCATm activity among the tissues examined. In mammalian tissues, the mitochondrial BCAT isoform predominates. Thus in the current study BCATm activity was determined in all tissues examined, except the brain. The cytosolic form (BCATc) is predominant in human and rat brain, placenta and ovaries (Sweatt et al, 2004). In the current study, brain homogenate was used for BCAT estimation due to lack of clear separation of cytosolic and mitochondrial fractions from brain tissue. Brain homogenate showed ~2.5μmol/min/mg protein BCAT activity (Table 6.1). However, whether the cytosolic or mitochondrial form is of predominance in the pig brain is not known. The significance of BCATc activity in rat brain and neuronal cells has been an area of interest recently (Yudkoff et al, 1996 and Lieth et al, 2001). Recently Sakai et al (2004) showed that ^{15}N labelled leucine contributed almost 50% of the glutamate nitrogen in glial and neuronal cells. Glutamate has been described as an excitatory neurotransmitter in the

central nervous system. Whether BCAA transamination plays a similar role in pig brain tissue is not known.

Developmental changes in BCATm activity have been reported in neonatal rats (Torres et al, 2001) and pre-ruminant sheep (Faure et al, 2001). Fetal rat liver BCATm activity is higher when compared to adult rat liver, and decreases rapidly following birth. Neonatal heart BCATm activity is lower than adult heart activity, and activity increases rapidly following birth and by day 21 reaches adult enzyme activity levels in rats (Torres et al, 2001). In pre-ruminant sheep skeletal muscle BCATm and BCATc activity decreased significantly from the fetus to new born, to the pre-ruminant stage (Faure et al, 2001). In the current study, however, pig tissues did not show any changes in BCAT activity during the early stages of postnatal development. BCATm activity in the small intestine increased from 0 day (0.78 ± 0.21μmol/min/mg protein) old to 7 day old (2.15 ± 1.11), though not significantly (Table 6.1). Interestingly, BCATm activity determined in adult pig enterocytes in the earlier study was 0.51 ± 0.01 μmol/min/mg protein (Chapter 5 – Table 5.1). This suggests that significant changes in BCATm activity in enterocytes occur between 7 days of age and maturity. Pig skeletal muscle and liver did not show any changes in BCATm activity during the first week of life. Heart BCAT activity decreased significantly from ~9 μmol/min/mg protein in neonates (Table 6.1), to 2.25 ± 0.11 μmol/min/mg protein during adulthood (Chapter 5 – Table 5.1).

Comparison of leucine and KIC oxidation rates is an indicator of whether the transamination or decarboxylation step is limiting oxidation of leucine. Furthermore, KIC oxidation gives a direct measure of the presence and activity of BCDH activity.

Oxidation of BCAA in enterocytes were low (0.1 – 0.3 nmol/mgprotein/hr) in all three age groups, compared with the oxidation of KIC (1.1 – 2.4 nmol/mg/hr) (Figure 6.4). KIC oxidation was ~ 85% higher than leucine oxidation, suggesting high BCDH activity relative to BCATm activity in enterocytes. Thus it appears that BCATm activity was limiting the catabolism of BCAA, in isolated intact neonatal enterocytes. Interestingly, oxidation of leucine was low in neonatal enterocytes (~0.2 nmol/mg protein/hr) compared to adult enterocytes (~ 4 nmol/mg protein/hr) (Chapter 5 - Figure 5.2) despite a significant decrease in BCATm activity in adult enterocytes compared to neonatal enterocytes. These data suggest that BCATm was not inhibiting BCAA flux through the catabolic pathway in adult pig enterocytes, but it was the limiting step in neonatal enterocytes.

BCAA oxidation in liver mitochondria was higher than BCAA oxidation in intact enterocytes (Figure 6.5 compared with Figure 6.4). KIC oxidation was once again higher than leucine oxidation, suggesting higher BCDH activity compared to BCAT activity in pig liver. BCDH activity also significantly decreases from birth to 7 days of age in pig liver (Figure 6.5). BCAA and KIC oxidation in muscle mitochondria interestingly, were significantly lower than BCAA oxidation in liver mitochondria during the first 3 days of life (Figures 6.5 and 6.6). At 7 days of age muscle BCAA and KIC oxidation rates were similar to liver oxidation rates. In muscle mitochondria leucine and KIC oxidation rates were similar, thereby suggesting that BCAT was not limiting BCAA oxidation. From the discussion above, in neonatal piglets, intact enterocytes and liver mitochondria have low BCAT and high BCDH activity when compared with muscle,

whereas muscle mitochondria have high BCAT and low BCDH activity when compared with enterocytes and liver.

No unique mechanism for the regulation of BCAT has been reported in any species. Thus, the rate of transamination of the BCAA primarily depends only on the concentrations of enzyme and the individual BCAA. The Michaelis-Menten constants (K_m) for BCAT in rats is ~ 0.4mM, whereas the K_m for BCDH is ~20μM (Chapter 1 – Table 1.2) (Harper et al, 1984). Thus, under conditions of normal feeding (excess of BCAA), an efficient system exists for removal of the luminally present BCAA by catabolizing excess BCAA to CO_2. And under post-absorptive conditions, the low BCAT in gut and liver would decrease the amount of ketoacids formed thereby sparing the BCAA for essential protein synthesis. The higher BCATm in muscle would ensure that muscle BCAA are transaminated to form glutamine and alanine to be released for transportation to the liver.

Leucine has been shown to stimulate both BCAT and BCDH activity in pre-ruminant lamb liver and jejunum (Papet et al, 1988), and stimulate BCDH activity in-vivo in rat skeletal muscle (Shimomura 2001and Aftring et al, 1986). BCAA concentrations are higher when compared to rest of the amino acids in sow milk (17.5 g of total BCAA/100g protein) and human milk (20.5 g of total BCAA/100g protein) (Reeds et al, 2000). Thus high concentrations of BCAA during feeding in the neonate, probably stimulates the BCAA enzymes, leading to significant catabolism of BCAA during first pass intestinal metabolism.

Stoll et al (1998) using portal mass balance estimates in milk fed piglets indicated that ~40% of the BCAA are utilized by the gut during first-pass metabolism,

and only 20% of the extracted BCAA was directed towards mucosal protein synthesis. In the current experiment, we have demonstrated that catabolism of BCAA to CO_2 occurs in enterocytes, thus establishing one of the fates of the enterally extracted BCAA. BCAA might also be contributing their nitrogen and carbon for the synthesis of glutamine and glutamate, key oxidative fuels for the small intestine. In human studies it has been shown using ^{15}N-Leucine infusion that 28% of the nitrogen in alanine (Harper et al, 1984) and 20% of the α-amino nitrogen in glutamine were derived from leucine. ^{15}N-Valine or leucine infusion in humans was also shown by Golden et al (1981) to cause increased enrichment of the amide-nitrogen of glutamine. In rats, leucine C-2 was incorporated into glutamine after the infusion of L-[1,2-^{13}C]-Leucine (Yoshida et al, 1991).

Enterocytes produced during early postnatal growth are structurally different from those produced later. Structural changes are thought to alter amino acid transport rates across the small intestine. Uptake of alanine has been shown to decrease during the first week of life in neonatal rats (Thomson & Keelan, 1986). In the current study, enterocytes showed structural changes during the first week of life. Spherical and lumped enterocytes were observed in 0 day pigs (Figure 6.1), whereas enterocytes isolated from 7 day old pigs were observed to be narrow and columnar, which is the commonly observed enterocyte of adult animals (Figure 6.3). Although structural changes were observed in neonatal porcine enterocytes, BCAA and KIC oxidation rates did not vary between 0 and 7 day old pigs and suggests that BCAA catabolizing enzymes are developed prenatally in the small intestine. The presence of BCAA enzymes in enterocytes during early stages of postnatal growth probably helps to provide energy to

the rapidly growing small intestine and to adapt quickly to changes taking place during the first week of life.

The fractional rates of protein synthesis in the gut and skeletal muscle differ significantly during adulthood, but in the young animal during early postnatal growth the growth constants are similar (Reeds et al, 1993). Thus the relative demands of gut and muscle influence the efficiency with which dietary energy (or in other words - carbon) and protein are utilized. Reeds et al (1993) also suggested that it appears that the gut and skeletal musculature are competitors for nutrients, when in fact this might be a regulated collaboration in which the maintenance of the organism as a whole is the ultimate goal. We suggest that a similar collaboration between the splanchnic organs (especially small intestine) and muscle to regulate BCAA metabolism during the critical postnatal stage is occurring to conserve BCAA for protein synthesis in muscle and to provide essential fuel for rapid growth in the small intestine.

In conclusion, during early postnatal development the neonatal enterocytes catabolize BCAA to CO_2. Significant oxidation of KIC in enterocytes suggests high BCDH activity in the small intestine and that, in-vivo rates of BCAA catabolism (oxidation) during first pass intestinal metabolism are high. Determining BCAA enzyme activity and oxidative rates in enterocytes isolated from piglets during weaning would be crucial to determine whether BCAA play a significant role during the next important stage of gut adaptation.

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7.0 SUMMARY, GENERAL DISCUSSION AND FUTURE DIRECTIONS

The first objective of this thesis was to compare the requirements for branched-chain amino acids (BCAA), in neonatal populations being fed either parenterally or enterally. The implications from the results obtained are two fold. First, during parenteral nutrition since the exact requirements for amino acids are not known, parenteral formulas tend to follow the amino acid composition of reference proteins, such as human milk. This has the possibility of either overfeeding or underfeeding an individual amino acid. An "optimal" profile for neonatal parenteral nutrition that provides amino acids in a combination to maximize protein accretion and growth, and minimize amino acid degradation has not been established. Second, during enteral nutrition, indispensable amino acid utilization by the small intestine has been reported; this could represent a net loss of amino acids to the rest of the body. Therefore determining the exact fate and quantity of amino acid being catabolized in the small intestine would help formulate amino acid solutions specific for each route of feeding. The research presented in this thesis has demonstrated that there is a significant impact of small intestinal catabolism on BCAA requirements.

In the first experiment, the total BCAA requirement during parenteral and enteral routes of feeding was determined in neonatal piglets using the indicator amino acid oxidation technique (IAAO). The ratio among the BCAA during both parenteral and enteral routes of feeding was maintained at 1:1.8:1.2 (isoleucine/leucine/valine), based on current oral dietary recommendations for 3-5 kg pigs (NRC, 1998). Furthermore, the ratio of 1:1.8:1.2 (isoleucine/leucine/valine) is very similar to the ratio in human milk (1:1.8:1)

and human fetal tissue (1:2.1:1.3), sow milk (1:2.4:1.4) and piglet fetal tissue (1:2.3:1.5) (Elango et al, 2002: Chapter 3 – Table 3.3).

The BCAA display antagonism, where increased or decreased intake of an individual BCAA has an antagonistic effect on the metabolism of another BCAA. By maintaining the same ratio among BCAA we intended to minimize potential antagonistic effects, which might skew our requirement estimate. Furthermore, we were able to directly compare the total BCAA requirements between routes of feeding at a constant ratio. To the best of our knowledge this approach to determine BCAA requirements is unique and has not been employed previously. The mean parenteral total BCAA requirement, as determined by the breakpoint of the two-phase regression crossover model, was found to be 1.53 g/kg/ d. The mean enteral requirement was estimated to be 2.64 g/kg/d. Therefore, the parenteral requirement of BCAA is 56% of the enteral requirement. This is a key and novel finding. Thus, in current neonatal parenteral formulas the total BCAA concentration is in excess and needs to be re-defined. The situation is exacerbated in premature infants being parenterally fed, where the amino acids may cause an excess nitrogen load on the immature metabolic system. The enteral requirement for total BCAA determined by the IAAO method was also ~30% higher than current NRC (1998) recommendations. This significantly higher requirement during enteral feeding compared to parenteral feeding suggested that BCAA were being utilized by the small intestine.

The plasma amino acid response to graded intakes of total BCAA at a fixed ratio, differed significantly due to route of feeding (Elango et al, 2002: Chapter 3 – Figures 3.2 and 3.4). Parenteral feeding resulted in a typical response to graded intakes of

a deficient amino acid: the plasma concentrations of the 3 deficient BCAA remained low until the requirement was met and then all three BCAA concentrations continued to increase with increasing intakes of BCAA above requirement (Chapter 3 - Figure 3.2). However, enteral feeding resulted in different responses for the individual BCAA. Leucine followed a pattern typical of a limiting amino acid; plasma concentrations remained low until requirement was reached, and once the total BCAA requirement was met, leucine concentrations started to increase. However, the plasma concentrations of isoleucine and valine were high, even when the supply of total BCAA in the diet was most deficient and increased with each increasing supply of total BCAA. Valine concentrations appeared to reach a plateau once the total BCAA requirement was reached (Chapter 3 - Figure 3.4) but isoleucine concentrations did not appear to plateau. This varied response in plasma amino acid concentrations due to route of feeding suggested that the ratio among the BCAA would be different and specific for parenteral and enteral feeding. Thus, the second study was designed to investigate whether the ratio among the BCAA could be improved upon for the two routes of feeding.

The IAAO method was once again used in the second experiment to determine whether the ratio among BCAA could be improved. Neonatal piglets were fed at 75% of mean total BCAA requirement, as determined in the first experiment for each of the parenteral and enteral routes of feeding; and the oxidation of the indicator amino acid, phenylalanine was measured. Change in oxidation of phenylalanine was then determined due to supplementation of isoleucine, leucine or valine to meet 100% of total BCAA requirement. Addition of the most limiting amino acid among the BCAA was hypothesized to decrease phenylalanine oxidation significantly. In the enterally fed

piglets, addition of any of the individual BCAA decreased phenylalanine oxidation by ~1%. This suggested that at the mean total BCAA requirement, all the 3 BCAA were co-limiting for protein synthesis. Addition of the individual BCAA during parenteral feeding resulted in significant changes to phenylalanine oxidation. Supplementation of isoleucine, valine, and leucine decreased oxidation of phenylalanine by ~13%, ~7% and ~2%, respectively (Elango et al, 2004: Chapter 4 – Figures 4.4). This suggests that isoleucine and valine were first and second limiting for whole body protein synthesis during parenteral feeding, when BCAA are provided at 75% of total BCAA requirement and a ratio of 1:1.8:1.2 (isoleucine/leucine/valine). An improved ratio among BCAA during parenteral feeding is thus suggested to be 1:1:1 (isoleucine/leucine/valine). This finding is quite significant and adds to the current literature which consistently shows that current parenteral and enteral formulas are inappropriate and should be designed specifically for each route of feeding. During enteral feeding, gut metabolism alters the composition and concentration of BCAA appearing in the portal vein. Parenteral feeding by-passes first pass gut metabolism, and the peripheral circulation receives BCAA unchanged. Based upon the significant differences in plasma BCAA concentrations due to route of feeding in the first experiment and the different ratios among BCAA during parenteral and enteral feeding in the second experiment, it appears that the small intestinal utilization of the individual BCAA would be different.

A comparison of the mean individual, and total BCAA requirements during parenteral and enteral feeding, with current NRC swine (1998) estimates reveals significant differences (**Table 7.1**). The total BCAA requirement of 17.6 g/100 g protein as determined in enterally fed 3 kg piglets is 63 % higher than the 10.8 g/100 g protein

estimate from NRC (1998). This indicates that the NRC (1998) estimates may be an under estimate and should be confirmed by additional direct observations rather than extrapolation from older pigs. The individual BCAA estimates from enterally fed piglets are quite similar to the DRI recommendations and the BCAA ratio is very similar. Whereas, during parenteral feeding the total BCAA requirement determined in piglets and the ratio among BCAA were significantly different from all other reported estimates (Table 7.1). These comparisons are very important. The similarity in the enteral requirements, other than NRC (1998), provides confidence in the recent DRI estimates. FAO/WHO (1985) and the DRI (2002) recommendations are based on nitrogen balance studies or factorial calculations based on human milk intake. Direct experiments need to be conducted to determine BCAA requirements in human neonates, using the minimally invasive IAAO technique, to confirm these values.

BCAA concentrations in plasma are lowered significantly, with increased aromatic amino acid (AAA) concentration under conditions of metabolic stress, hepatic encephalopathy and chronic renal failure. This altered AAA/BCAA ratio has been implicated to exacerbate the catabolic state. Thus many clinical studies have been conducted over the past 3 decades to normalize plasma BCAA concentrations by supplementing BCAA or leucine alone. Results from these studies have been conflicting and controversial (Teasley & Buse, 1989 and Vente et al, 1991). The studies have used varied concentrations and ratios of BCAA, as well as different routes of infusion. From the first two experiments presented in this thesis, it is clear that BCAA supplementation studies during parenteral and enteral nutrition should be conducted not only at specific BCAA concentrations but also at the appropriate ratio among BCAA.

Table 7.1 Comparison of branched-chain amino acid (BCAA) requirements in piglets and human infants

Amino Acid	Enterally Fed Piglets[1]	Parenterally Fed Piglets[2]	NRC Swine (1998)[3]	FAO/WHO/ UNU (1985)[4]	DRI Committee (2002)[5]
	g/100 g protein				
Isoleucine	4.4	3.4	2.5	3.2	4.0
Leucine	7.9	3.4	4.6	7.3	7.1
Valine	5.3	3.4	3.0	4.2	4.0
Ratio (ile/leu/val)	1:1.8:1.2	1:1:1	1:1.8:1.2	1:2.3:1.3	1:1.8:1
Total BCAA	17.6	10.2	10.8	14.7	15.1

[1]Requirement estimates and ratio from IAAO studies (Chapter 3 and 4)

[2] Requirement estimates and ratio from IAAO studies (Chapter 3 and 4)

[3] Recommendations for 3-5kg piglets extrapolated from 10kg piglets

[4] Recommendations for 0-6 mo infants based on N balance studies

[5] Recommendations for 0-6 mo infants based on factorial calculations

The first and second experiments show that small intestinal metabolism of BCAA has a significant impact not only on the BCAA requirement estimates, but also on plasma BCAA concentrations. Metabolism of BCAA by the small intestine could involve extensive transamination to branched-chain α-ketoacids (BCKA) with the ketoacids released into portal circulation for further utilization by the liver, as shown in adult humans (Matthews et al, 1999). However, the IAAO technique uses the oxidation of the indicator amino acid (phenylalanine), as an indicator for whole body protein synthesis, and only when the whole body requirement is met for BCAA, including the BCKA, does the oxidation of phenylalanine attain plateau and remain constant. Thus based upon the indicator oxidation data presented, the gut must be catabolizing BCAA. Leucine utilization by the splanchnic organs have been shown in various species with the help of in-vivo tracers, such as in dogs (Yu et al, 1990 and 1995), sheep (Papet et al, 1988), humans (Gelfand et al, 1986) and piglets (Stoll et al, 1998). But little or no data exists on BCAA enzymes in the small intestine. Furthermore, whether the enterocytes could oxidize essential amino acids was unknown. Thus, the final two experiments examined BCAA catabolic enzymes and oxidation in enterocytes.

The third experimental series was designed to develop in-vitro procedures for isolating enterocytes from porcine intestine, and optimize the branched-chain aminotransferase (BCAT) assay and in-vitro oxidation assay. Enterocytes were successfully isolated from small intestine of 120kg pigs. They were >90%viable, allowing us to conduct enzyme and oxidation assays. BCAA catabolism is regulated by 2 key enzymes: first, the transaminase enzyme, branched-chain aminotransferase (BCAT) and second, the decarboxylating enzyme, branched-chain dehydrogenase (BCDH). BCAT

has been shown in most mammals to exist as two isoforms - BCAT mitochondrial (BCATm) and cytosolic (BCATc). BCATm is the most predominant isoform in human and rat tissues. Heart BCATm has the highest activity of any tissues and therefore is a good comparison tissue for BCAA enzyme studies (Hutson 1988). Thus, BCATm activity was measured in pig heart, liver and enterocyte mitochondria. Heart mitochondria had the highest BCATm activity in growing pigs, and when expressed as a percentage, enterocyte was 20% and liver was 60% of heart BCATm activity (Chapter 5 – Table 5.1). BCATm specific activity has been reported to be negligible in rat small intestinal mucosa (Suryawan et al, 1998). Recently, Burrin et al (2003) in 28 day old parenterally or enterally fed piglets, measured BCAT and BCDH activity in small intestinal tissues that had been frozen. They reported ~140 mU BCAT activity/min/g tissue in enterally fed, compared to ~110 mU BCAT activity in parenterally fed piglets. BCDH activity was reported to be ~5.3 mU/min/g tissue and ~3.2 mU/min/g in enterally and parenterally fed piglets, respectively. These data on frozen tissues should be interpreted with caution until there is clear evidence whether or not freezing alters the activity of the BCAA enzymes. We are presently unaware of any data comparing BCAT and BCDH in fresh or frozen enterocytes. The results from the series of experiments presented here are the first report that freshly isolated enterocyte mitochondria possesses the transaminase at significant levels. Therefore the first step in BCAA catabolism could potentially occur in the pig small intestine.

In-vitro oxidation studies were subsequently conducted in intact enterocytes and liver mitochondria using 1-^{14}C-isoleucine, leucine and valine to determine whether catabolism of BCAA to CO_2 occurs in the small intestine and liver.

Oxidative rates of 1-^{14}C-ketoisocaproic acid (KIC) were also measured to determine whether transamination or decarboxylation steps were limiting oxidation. Furthermore, KIC oxidation gives a direct measurement of the presence and activity of the BCDH enzyme. Leucine and KIC oxidation were significantly higher than valine and isoleucine oxidation in intact enterocytes (Chapter 5 – Figure 5.2). KIC oxidation in liver mitochondria was significantly higher than all BCAA (Chapter 5 – Figure 5.3). Thus it appears, in adult pig enterocytes there is a preferential oxidation of leucine, compared to isoleucine and valine. Furthermore, BCATm activity is not limiting leucine oxidation in enterocytes because the oxidative rates of leucine and KIC were similar. In liver mitochondria, BCATm is limiting BCAA oxidation, as indicated by the significantly higher oxidation of KIC compared to the BCAA. Nevertheless, BCAA oxidation in enterocytes was shown to occur for the first time.

The initial BCAA requirement studies were conducted in ~7 day old piglets. Whether the neonatal small intestine can oxidize BCAA was unknown. The small intestine undergoes significant changes immediately after birth, as the route of nutrition changes from a predominantly intravenous route in-utero to oral feeding after birth. Thus the final experiment was designed to examine whether neonatal enterocytes can oxidize BCAA. Furthermore, to examine early postnatal changes in BCATm activity and BCAA oxidation, enterocytes, liver and muscle mitochondria from 0, 3 and 7 day piglets were studied. BCATm activity in enterocytes was higher in 7 day than 0 day old piglets, although not significantly (Chapter 6 – Table 6.1). Isoleucine, leucine and valine oxidation were unaffected by postnatal age and were low in neonatal enterocytes, whereas KIC oxidation was ~85% higher than leucine oxidation (Chapter 6 – Figure 6.4).

Thus, it is clear that BCDH activity is significantly higher than BCATm activity in neonatal enterocytes. BCATm enzyme activity regulation is via enzyme concentration and individual BCAA concentrations. The Michaelis-Menten constants (K_m) for BCATm in rats have been reported to be ~0.4mM, whereas the K_m for BCDH is ~20μM (Chapter 1 – Table 1.2). This suggests that under conditions of feeding in-vivo, when the luminal concentrations of BCAA are higher than in the fasted state, BCAA will be converted to BCKA in the enterocytes and subsequently oxidized to CO_2.

To compare oxidation rates between gut (small intestine) and liver, intact enterocyte leucine oxidation was converted to mitochondrial oxidative rates (**Table 7.2**). The mitochondrial oxidative rates from both enterocytes and liver were also extrapolated to whole organ basis based on calculations described in Table 7.2. In the gut, oxidation of leucine increased 5 fold from birth to 7 days of age. There was a 3000 fold increase in gut leucine oxidation from birth to adulthood. When 7d gut was compared to adult gut oxidation, there was a 650 fold increase. Oxidation of leucine in the liver also increased 5 fold during the first week, similar to the gut. But comparison of leucine oxidation in liver between birth and adulthood gave a 77 fold increase, and a 14 fold increase between 7 d old and adulthood. The whole organ increase in oxidation in the gut is due to an increase in oxidative rate, whereas in the liver it is due to increasing liver weight with age (Actual calculation values described in Appendix 8.5, Table 8.5.1). The actual oxidative rate in mg mitochondrial protein per hour is lower in the neonatal gut relative to liver. However, at adulthood, the potential to oxidize leucine is higher in the small intestine than in the liver, as observed by similar oxidative rates; 21.5 versus 27.5 nmol oxidation/mg/hr, in gut and

Table 7.2 Comparison of leucine oxidation between small intestine and liver[1]

Tissue	0 Day	3 Day	7 Day	Adult
		µmol oxidation / organ[4] / day		
Gut[2]	1.9	2.5	9.1	5878
Liver[3]	519	2060	2759	40275

[1]Data calculation as follows:

[2]Gut:

Intact enterocyte oxidation rate was converted to mitochondrial values by multiplying by 5 (3.6 / 17.9, intact enterocyte oxidative rate/ enterocyte mitochondrial oxidative rate), based on Figure 5.5, Chapter 5.

$$\left[\frac{\text{Mitochondrial yield (mg)}}{\text{Wt of gut used (g)}} \times \text{Avg entire small intestine wt (g)}\right] \times \text{Oxidation rate (nmol/mg mito pr/hr)}$$

= [leucine oxidation in entire small intestine/hr] X 24 hrs = potential small intestinal leucine oxidation per day

[3]Liver:

$$\left[\frac{\text{Mitochondrial yield (mg)}}{\text{Wt of liver used (g)}} \times \text{Average liver wt (g)}\right] \times \text{Oxidation rate (nmol/mg mito pr/hr)}$$

= [leucine oxidation in liver/hr] X 24 hrs = potential liver leucine oxidation per day

[4]Average gut and liver weight for 0, 3 and 7 day old piglets were determined in Experiment 4 (Chapter 6); Gut d0 = 85g, d3 = 150g, d7 = 163.5g; Liver d0 = 42g, d3 = 85g, d7 = 95g.

[4]Average gut weight (6kg) and liver weight (2.5kg) for adult pigs was based on Cranwell (1995) and Pond and Houpt (1978).

Actual numbers used for calculations are provided in Table 8.5.1 (Appendix)

liver, respectively. The implication of these calculations is that, potential leucine oxidation in the intestine is low at birth, develops rapidly in the postnatal period and is similar to the liver in adult pigs. This suggests that leucine oxidation must have some important function, otherwise the potential oxidation would not be so high. Clearly the question of the reason for this high potential leucine oxidation demands further examination. There may also be an important function of leucine oxidation in growth and development of the small intestine. Examination of KIC versus leucine oxidation could reveal whether the oxidation is to provide carbon for energy or nitrogen for the synthesis of other metabolites.

In the final experiment, comparison of BCAA and KIC oxidation among enterocytes, liver and skeletal muscle demonstrated inter-organ differences in neonates in BCAA catabolism. In intact enterocytes and liver mitochondria BCAA oxidation was lower than KIC oxidation, indicating that enterocytes and liver have higher BCDH activity compared to BCAT activity. Oxidation of BCAA and KIC in muscle mitochondria were similar suggesting lower BCDH activity compared to BCAT. Thus, under conditions of normal feeding (excess of BCAA), an efficient system for removál of the excess BCAA exists whereby the luminally present BCAA are catabolized to CO_2. In contrast under post-absorptive conditions, the low BCAT in gut and liver would serve to decrease the amount of ketoacids formed, thereby sparing the BCAA for essential protein synthesis. The higher BCATm activity compared to BCDH activity in muscle would ensure that muscle BCAA are transaminated to form glutamine and alanine to be released for transportation to the liver. In the first in-vivo experiment, the BCAA requirement during enteral feeding was significantly higher than parenteral feeding, demonstrating

BCAA utilization by the small intestine. The final two in-vitro experiments clearly showed that the enterocytes possess the necessary enzymes to catabolize BCAA to CO_2. The quantity of BCAA oxidized in-vivo during first pass intestinal metabolism appears to be significant. A stoichiometric calculation, assuming complete catabolism of the BCAA carbons to CO2 and the pathways shown in Figure 1.1 and 1.3 (Chapter 1), revealed interesting results. The complete oxidation of a molecule of leucine will yield 40 ATP, isoleucine yields 13 ATP and valine yields 16 ATP. In comparison, complete oxidation of glucose yields 36 or 38 ATP. Under conditions of adequate protein and calorie intake, the gut spares glucose, and does not use it as the predominant oxidative fuel (Reeds et al, 2000b). Thus, leucine could spare glucose for energy in the small intestine almost mole for mole, under standard conditions. In terms of ATP, acetyl CoA produces 12 ATP by complete oxidation, and catabolism of leucine produces acetylCoA and acetoacetate. Acetoacetate, a ketone body, is significant because its catabolism yields 2 acetyl CoA; and is used in tissues such as heart muscle and renal cortex, as an energy source, in preference to glucose. Acetoacetate, derived from BCAA, may be used in a similar manner or be a preferred substrate by the small intestine. This hypothesis deserves further research.

During enteral nutrition for a 3 kg piglet receiving 15 g protein/kg/day, at a mean total BCAA requirement of 2.64 g/kg/d, leucine, isoleucine and valine intake would be 27, 15 and 20 mmol/d, respectively. If 45% of BCAA intake was used by the intestine, as suggested by the data in Chapter 3, then gut utilization of total BCAA would mean 12, 7 and 9 mmol/d of leucine, isoleucine and valine, respectively could be providing significant ATP synthesis in the small intestine. Thus BCAA could be a very

important energy source for the intestine. Future experiments need to be designed to quantitatively determine the contribution of enterally supplied BCAA to CO_2 production and thus to the energy needs of the gut. One approach would be to use tracers. BCAA tracers could be infused to include gut metabolism (intragastric infusion) and exclude gut metabolism (intraportal infusion) with collection of CO_2 derived from BCAA by the intestine. The results from such studies could have significant implications in neonatal nutrition support, especially during gastrointestinal disorders.

Necrotizing Enterocolitis (NEC) is an acquired gastrointestinal disorder often observed in premature infants (Guthrie et al, 2003). Although the etiology for NEC is yet unknown, enteral feeding and bacterial translocation appear to be significant risk factors. Incidence of short bowel syndrome in children has been reported to be common in neonates who survive NEC (Vennarecci et al, 2000). In such conditions the neonatal nutrition support depends upon parenteral feeding. Parenteral nutrition on the other hand has been associated with liver cholestasis. TPN induced-choleostasis is thought to occur due to direct toxicity of TPN on the liver and, hepatic nutritional deficiencies resulting from the nutritional inadequacies of TPN (Sandhu et al, 1999). Thus formulation of BCAA supplemented diets to optimize fuel supply to the small intestine, under conditions of parenteral and enteral feeding could alleviate some of the problems discussed above, associated with delivering nutrition support.

Maple syrup urine disease (MSUD) is an autosomal recessive disorder caused by defects in BCDH enzyme. It results in increased BCAA and BCKA concentrations in plasma and other body fluids (Baulny & Saudubray, 2002). In neonates increased BCAA and BCKA concentrations could potentially cause permanent

neurological damage. Treatment involves dietary manipulation of BCAA intake to maintain plasma BCAA concentrations below toxic levels. In adult MSUD, the estimation of BCAA intake is normally based on deducting the obligatory oxidation of BCAA from the requirement for BCAA for healthy subjects. Recently, in adults, Riazi et al (2004) estimated the total BCAA requirement in MSUD patients directly using the IAAO technique. It was shown that the total BCAA requirement was 45mg/kg/d, when compared to the predicted requirement of 85 mg/kg/d. However, the BCAA requirements in neonatal MSUD patients requiring parenteral nutritional support are unknown. Direct BCAA requirement studies in neonates comparing healthy and MSUD patients is necessary to decrease long term complications associated with inappropriate BCAA intakes.

In the swine industry early weaning is becoming more popular and improved weaning diet formulations are critical to alleviate weaning-induced gut stress. Gastrointestinal disorders, such as diarrhea are common and lead to overall decreased productivity. BCAA supplementation could provide significant energy to the small intestine of these early-weaned pigs and needs to be examined.

Comparison of sow milk amino acid composition and piglet tissue mixed protein amino acid composition reveals interesting correlations. Leucine and isoleucine concentrations in milk, 8.9 g/100g protein and 3.6 g/100g protein, respectively, exceed their needs for protein deposition. However, milk concentrations of arginine (4.4 g/100g protein), alanine (3.6 g/100g) and glycine (3.2 g/100g) are far less than the concentration (6.9, 6.6 and 9.7 g/100g protein, respectively) found in neonatal piglet tissue (Davis et al, 1994 and Wu & Knabe, 1994). BCAA may, therefore, be providing necessary carbons

and nitrogen in the gut for the synthesis of these deficient amino acids. Arginine synthesis in the neonatal piglet has been shown to be dependent on the gut and gut synthesis of arginine provides up to 50% of the arginine requirement during enteral feeding (Bertolo et al, 2003). P-5-C synthase activity has been found to be low in intestinal tissues, and therefore arginine synthesis from glutamate and glutamine is not considered to be significant. However, the in-vivo contribution of glutamate to arginine synthesis is still unknown. Nevertheless, it has been shown that almost all enteral glutamate is catabolized during first pass-intestinal metabolism (Reeds et al, 2000a) and since leucine has been shown to increase glutamate synthesis endogenously (Harper et al, 1984), the possibility for BCAA carbons and nitrogen to be involved in arginine synthesis exists. Mitochondrial compartmentation of the BCAA enzymes and carbamoyl phosphate synthetase I (Davis & Wu, 1998) might also play a role in arginine synthesis. Transamination of BCAA could increase ammonia synthesis intramitochondrially and provide for increased citrulline synthesis from carbamoyl phosphate. BCAA are significant nitrogen donors in skeletal muscle for alanine synthesis (Harper et al, 1984). Because significant catabolism of BCAA in the enterocytes occurs, this provides increased nitrogen for transamination reactions and alanine synthesis. Thus in neonates, BCAA could potentially be involved in the synthesis of these dietary dispensable amino acids, and future studies need to confirm the contribution of BCAA carbons and nitrogen for arginine and alanine synthesis in the gut. ^{15}N labeled BCAA tracer studies need to be designed to examine the contribution of BCAA to biosynthesis of alanine, arginine and glycine in the small intestine.

BCAA, especially leucine, have been implicated to provide its carbon (Yoshida et al, 1991) and α-nitrogen (Harper et al, 1984) in rat skeletal muscle for glutamine synthesis. Golden et al (1981), using ^{15}N-labeled tracers in humans, observed that the amide-nitrogen of glutamine could also be donated from valine and leucine. This suggests that nitrogen produced from BCAA catabolism provides for glutamine synthesis via the glutamate dehydrogenase reaction. Whether the same occurs in the small intestine is not yet known. BCAA involvement in small intestinal glutamine synthesis could have important implications in nucleic acid synthesis. Nucleic acids turnover rapidly in developing small intestinal cells (Uauy-Dagach & Quan, 1994). Furthermore, purine synthesis in-vivo is dependent on the glutamine carbon as well as the amide-nitrogen. However, nucleic acid synthesis from amino acids or glucose is metabolically expensive. Therefore the small intestine has been postulated to depend more on the salvage pathway for the synthesis of purine and pyrimidine bases from degradation of RNA and DNA (Yu, 2002). In parenteral and enteral formulations, often no nucleotides exist and thus under conditions of prolonged nutrition support, amino acids must play a significant role in nucleic acid synthesis. Future work needs to be conducted to examine the role of dietary amino acids in nucleic acid synthesis in the intestine of the rapidly growing neonate receiving formulations lacking nucleic acids.

The series of experiments described in this thesis have clearly demonstrated that BCAA metabolism is altered significantly by route of feeding. Specifically, the parenteral requirement of total BCAA is 56% of the enteral requirement. The route of feeding also affects the ratio among the BCAA, and the ratio of 1:1.8:1.2 (isoleucine/leucine/valine) as found in milk proteins is appropriate during enteral feeding.

During parenteral feeding the new ratio suggested is 1:1:1 (isoleucine/leucine/valine). These findings have important implications in the nutrition support of neonates who have to be nourished during a critical period of rapid development. The significantly higher BCAA requirements during enteral feeding compared to parenteral feeding is due to BCAA utilization by the small intestine. The in-vitro studies provide biochemical evidence for the complete catabolism of BCAA to CO_2. These findings are also crucial because oxidation of the dietary indispensable BCAA during first-pass intestinal metabolism would make a portion of the BCAA unavailable to the rest of the body for necessary protein synthesis. Conversely, BCAA could provide a significant source of energy to the rapidly growing small intestine, especially under conditions of gastrointestinal disease or stress, and provide nitrogen for the biosynthesis of arginine, glutamine, glutamate, glycine and alanine.

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8.0 APPENDIX

8.1 Diet Formulations

Table 8.1.1 Amino acid composition of elemental diets

Amino Acid	Concentration (mg/g total amino acids)
Histidine	31
Isoleucine	45
Leucine	103
Lysine	70
Methionine	19
Phenylalanine	40
Threonine	52
Tryptophan	21
Valine	52
Cysteine	14
Tyrosine	27
Alanine	104
Arginine	78
Aspartate	60
Glutamate	103
Glycine	38
Proline	82
Serine	46
Taurine	5
Total	1000 mg

Table 8.1.2 Nutrient profile of complete elemental diets

Nutrient	Concentration as fed (/L)
Alanine	3.29 g
Arginine	1.88 g
Aspartate	1.88 g
Cysteine	0.45 g
Glutamate	3.25 g
Glycine	0.76 g
Glycyl-L-Tyrosine	0.72 g
Histidine	0.96 g
Isoleucine	1.43 g
Lysine	2.19 g
Methionine	0.60 g
Phenylalanine	0.98 g
Proline	2.56 g
Serine	1.73 g
Taurine	0.14 g
Threonine	1.64 g
Tryptophan	0.66 g
Tyrosine	0.24 g
Valine	1.64 g
Dextrose	50.55 g
K_2HPO_4	0.88 g
KH_2PO_4	0.61 g
Potassium acetate	0.82 g
NaCl	1.21 g
$MgSO_4$. ($7H_2O$)	0.44 g
ZnSO4. ($7H_2O$)	0.05 g
$MnSO_4$. (1.65% w/v)	73.5 µL
Calcium gluconate	3.58 g
Trace element mix [a]	9.2 mL
MVI vitamin mix [b]	9.2 mL
Fe dextran (8% v/v)	3.1 mL
Intralipid 20%[c]	97.5 mL
Water (ddH_2O)	Complete to 1L

Footnotes: a, b, c – refer following page

[a]Contents of Trace element mix

Trace Element	Concentration (µmol/mL)
Chromium (chromium chloride)	0.076
Copper (copper sulfate)	6.3
Iodine (Sodium iodide)	0.47
Manganese (manganese sulfate)	1.8
Selenium (selenious acid)	0.25
Zinc (zinc sulfate)	44.9

Formulation based on Micro +6 ® (Sabex, Boucherville, QC)

[b]Contents of MVI Pediatric (multi-vitamin for intravenous infusions)

Vitamin	Concentration
Vitamin A	2300 U
Thiamine (as hydrochloride)	1.2 mg
Riboflavin (as phosphate)	1.4 mg
Niacinamide	17.0 mg
d-pantothenic acid	5.0 mg
Pyridoxine hydrochloride	1.0 mg
Biotin	20 µg
Folic acid	0.14 mg
Vitamin B_{12}	1 µg
Ascorbic acid	80 mg
Vitamin D	400 U
Vitamin E	7.0 U
Vitamin K_1	0.2 mg

MVI Pediatric® is a registered trademark of aaiPharma Inc, NC

Contents of Intralipid 20% intravenous fat emulsion

Constituent		Concentration
Soybean oil		20%
Linoleic acid	52%	
Linolenic acid	8%	
Oleic acid	22%	
Palmitic acid	13%	
Stearic acid	4%	
Egg phospholipids		1.2%
Glycerin (anhydrous)		2.2%

Intralipid 20%® is a registered trademark of Fresenius Kabi, Germany

8.2 SAS Program to calculate BCAA requirement breakpoint

The following SAS program combines phenylalanine oxidation data (as a percentage of dose) from both parenterally (IV) and enterally (IG) fed piglets and calculates the breakpoint estimate. This program is different from previously used breakpoint programs because it combines data from two different treatments (IV and IG) and also determines whether the two breakpoints are significantly different ($P < 0.05$) from each other.

The model used: $Y = b0 + b1*x - b1*x1$

Where, b0=4 and b1=-4, thereby the program starts at 0. The values are randomly assigned, as this an iterative (trial and error) program. The breakpoint is denoted by 'knot'. x1=0, prior to the estimated breakpoint, and x1=(x - breakpoint), after the breakpoint.

```
/************************************************/

 /*       Two-phase Linear Regression Program     */
 /************************************************/

data new;
        input treat $ x y;
        cards;

iv      0.17    2.59
iv      0.18    5.39
iv      0.19    8.97
iv      0.20    1.52
iv      0.43    4.3
iv      0.46    5.96
iv      0.49    2.33
iv      0.49    3.27
iv      0.70    1.17
iv      0.71    1.2
iv      0.76    3.94
iv      0.79    2.19
iv      0.88    2.91
iv      1.00    2.43
```

```
iv      1.07    2.7
iv      1.08    2.21
iv      1.32    1.52
iv      1.34    1.13
iv      1.34    0.77
iv      1.36    0.64
iv      1.87    0.94
iv      1.88    0.7
iv      1.91    0.33
iv      1.96    0.28
iv      2.07    0.68
iv      2.30    0.16
iv      2.36    0.98
iv      2.38    0.59
iv      2.58    0.47
iv      2.69    0.52
iv      2.81    0.31
iv      3.03    0.76
iv      3.09    0.4
iv      3.19    0.38
iv      3.47    0.51
iv      3.61    0.1
ig      0.18    2.62
ig      0.18    2.85
ig      0.19    2.89
ig      0.21    5.07
ig      0.75    3.34
ig      0.77    2.25
ig      0.77    2.55
ig      0.78    2.85
ig      1.25    3.34
ig      1.25    1.61
ig      1.38    1.46
ig      1.38    1.92
ig      1.73    1.72
ig      1.77    1.82
ig      1.90    1.08
ig      1.93    0.92
ig      2.49    0.23
ig      2.50    0.44
ig      2.51    0.38
ig      2.73    0.29
ig      2.74    0.25
ig      3.11    0.48
ig      3.15    0.18
ig      3.34    0.33
ig      3.58    0.22
ig      3.64    0.13
ig      3.73    0.21
ig      3.76    0.34
;


title 'Two Regressions, First IV';
data new4;set new;if treat="ig" then delete;
proc nlin data=new4;
```

```
        parms b0=4 b1=-4 knot = .3;
                x1=max(0,x-knot);
                model y= b0 + b1*x -b1*x1;
        output out=cativ p=predic;
        run;
proc gplot data=cativ;
  plot (y predic)*x/overlay;
  by treat;run;
proc print data=cativ;
title 'Two Regressions, Next IG';


data new4;set new;if treat="iv" then delete;
proc nlin data=new4;
        parms b0=4 b1=-4  knot = .3;
                x1=max(0,x-knot);
                model y= b0 + b1*x -b1*x1;
        output out=catig p=predic;
        run;
proc gplot data=catig;
  plot (y predic)*x/overlay;
  by treat;run;
proc print data=catig;

data new5;set new;
  if treat = "ig" then d1=1;else d1=0;  *d1 is the dummy used to get
analysis for ig;
  if treat ="iv" then d2=1;else d2=0;    *d2 is the dummy used to get
analysis for iv;
  run;
quit;
```

8.2.1 SAS Output for BCAA requirement in piglets being fed parenterally and enterally

Two Regressions, First IV 1
21:00 Monday, April 12, 2004

The NLIN Procedure
Iterative Phase
Dependent Variable y
Method: Gauss-Newton

Iterations	b0	b1	knot	Error Sum of Squares
0	4.0000	-4.0000	0.3000	159.0
1	3.9916	-3.0375	0.4119	152.3
2	3.9854	-2.3179	0.5524	146.1
3	4.4470	-2.4830	0.9773	93.3480
4	5.2436	-3.5810	1.2577	53.6125
5	5.0539	-2.9025	1.4788	51.9412
6	5.1090	-3.0105	1.5301	50.8514
7	5.1092	-3.0110	1.5285	50.8511

NOTE: Convergence criterion met.

Estimation Summary

Method	Gauss-Newton
Iterations	7
Subiterations	5
Average Subiterations	0.714286
R	1.752E-6
PPC(knot)	7.744E-7
RPC(knot)	0.001061
Object	6.074E-6
Objective	50.85112
Observations Read	36
Observations Used	36
Observations Missing	0

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	3	197.2	65.7254	25.60	<.0001
Residual	33	50.8511	1.5409		
Uncorrected Total	36	248.0			
Corrected Total	35	129.8			

The NLIN Procedure

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
b0	5.1092	0.5824	3.9243	6.2942
b1	-3.0110	0.6845	-4.4037	-1.6184
knot	1.5285	0.2250	1.0708	1.9862

Approximate Correlation Matrix

	b0	b1	knot
b0	1.0000000	-0.8791244	-0.4981161
b1	-0.8791244	1.0000000	0.7887646
knot	-0.4981161	0.7887646	1.0000000

Obs	treat	x	y	PREDIC
1	iv	0.17	2.59	4.59737
2	iv	0.18	5.39	4.56726
3	iv	0.19	8.97	4.53715
4	iv	0.20	1.52	4.50704
5	iv	0.43	4.30	3.81451
6	iv	0.46	5.96	3.72418
7	iv	0.49	2.33	3.63384
8	iv	0.49	3.27	3.63384
9	iv	0.70	1.17	3.00153
10	iv	0.71	1.20	2.97142
11	iv	0.76	3.94	2.82087
12	iv	0.79	2.19	2.73054
13	iv	0.88	2.91	2.45954
14	iv	1.00	2.43	2.09822
15	iv	1.07	2.70	1.88745
16	iv	1.08	2.21	1.85734
17	iv	1.32	1.52	1.13469
18	iv	1.34	1.13	1.07447
19	iv	1.34	0.77	1.07447
20	iv	1.36	0.64	1.01425
21	iv	1.87	0.94	0.50687
22	iv	1.88	0.70	0.50687
23	iv	1.91	0.33	0.50687
24	iv	1.96	0.28	0.50687
25	iv	2.07	0.68	0.50687
26	iv	2.30	0.16	0.50687
27	iv	2.36	0.98	0.50687
28	iv	2.38	0.59	0.50687
29	iv	2.58	0.47	0.50687
30	iv	2.69	0.52	0.50687

31	iv	2.81	0.31	0.50687
32	iv	3.03	0.76	0.50687
33	iv	3.09	0.40	0.50687
34	iv	3.19	0.38	0.50687
35	iv	3.47	0.51	0.50687
36	iv	3.61	0.10	0.50687

The NLIN Procedure
Iterative Phase
Dependent Variable y
Method: Gauss-Newton

Iter	b0	b1	knot	Sum of Squares
0	4.0000	-4.0000	0.3000	93.6737
1	3.8794	-3.3607	0.3210	93.6693
2	3.8070	-2.9762	0.3384	93.5561
3	3.7626	-2.7412	0.3518	93.4121
4	3.7080	-2.4513	0.3712	93.3684
5	3.6744	-2.2732	0.3859	93.2762
6	3.6328	-2.0528	0.4070	93.2704
7	3.6072	-1.9170	0.4228	93.2105
8	3.5913	-1.8327	0.4340	93.1430
9	3.5716	-1.7279	0.4492	93.0916
10	3.5470	-1.5976	0.4704	93.0880
11	3.5318	-1.5169	0.4857	93.0486
12	3.5128	-1.4164	0.5067	93.0442
13	3.5011	-1.3540	0.5216	93.0118
14	3.4864	-1.2763	0.5419	93.0037
15	3.4773	-1.2279	0.5561	92.9757
16	3.4659	-1.1677	0.5751	92.9635
17	3.4588	-1.1301	0.5882	92.9386
18	3.4500	-1.0833	0.6056	92.9231
19	3.4445	-1.0541	0.6173	92.9007
20	3.4377	-1.0177	0.6329	92.8833
21	3.4291	-0.9723	0.6536	92.8800
22	3.4237	-0.9440	0.6677	92.8634
23	3.4171	-0.9087	0.6864	92.8551
24	3.4129	-0.8867	0.6990	92.8395
25	3.4077	-0.8591	0.7155	92.8284
26	3.4045	-0.8420	0.7264	92.8140
27	3.4005	-0.8206	0.7406	92.8016
28	3.3954	-0.7938	0.7593	92.7872
29	3.3953	-0.7922	0.7643	92.5592
30	3.3945	-0.7842	0.7893	91.4664
31	3.4002	-0.7931	0.8881	85.4974
32	3.4249	-0.8321	1.3079	61.7703
33	3.4568	-0.8448	2.5442	21.3089
34	3.6978	-1.2819	2.6902	7.2569
35	3.7082	-1.3008	2.6425	7.2455
36	3.7082	-1.3008	2.6431	7.2455

The NLIN Procedure
Iterative Phase
Dependent Variable y
Method: Gauss-Newton

NOTE: Convergence criterion met.

Estimation Summary

Method	Gauss-Newton
Iterations	36
Subiterations	74
Average Subiterations	2.055556
R	5.154E-7
PPC(knot)	1.139E-7
RPC(knot)	0.000209
Object	8.757E-7
Objective	7.245475
Observations Read	28
Observations Used	28
Observations Missing	0

Source	DF	Sum of Squares	Mean Square	F Value	Approx Pr > F
Regression	3	101.6	33.8750	67.82	<.0001
Residual	25	7.2455	0.2898		
Uncorrected Total	28	108.9			
Corrected Total	27	46.5586			

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
b0	3.7082	0.2347	3.2248	4.1916
b1	-1.3008	0.1585	-1.6273	-0.9743
knot	2.6431	0.2377	2.1536	3.1326

The NLIN Procedure

Approximate Correlation Matrix

	b0	b1	knot
b0	1.0000000	-0.8503459	-0.3932607
b1	-0.8503459	1.0000000	0.7096588

knot -0.3932607 0.7096588 1.0000000

Two Regressions, Next IG 7
21:00 Monday, April 12, 2004

Obs	treat	x	y	PREDIC
1	ig	0.18	2.62	3.47405
2	ig	0.18	2.85	3.47405
3	ig	0.19	2.89	3.46104
4	ig	0.21	5.07	3.43502
5	ig	0.75	3.34	2.73258
6	ig	0.77	2.25	2.70656
7	ig	0.77	2.55	2.70656
8	ig	0.78	2.85	2.69355
9	ig	1.25	3.34	2.08217
10	ig	1.25	1.61	2.08217
11	ig	1.38	1.46	1.91306
12	ig	1.38	1.92	1.91306
13	ig	1.73	1.72	1.45777
14	ig	1.77	1.82	1.40574
15	ig	1.90	1.08	1.23663
16	ig	1.93	0.92	1.19761
17	ig	2.49	0.23	0.46914
18	ig	2.50	0.44	0.45614
19	ig	2.51	0.38	0.44313
20	ig	2.73	0.29	0.27000
21	ig	2.74	0.25	0.27000
22	ig	3.11	0.48	0.27000
23	ig	3.15	0.18	0.27000
24	ig	3.34	0.33	0.27000
25	ig	3.58	0.22	0.27000
26	ig	3.64	0.13	0.27000
27	ig	3.73	0.21	0.27000
28	ig	3.76	0.34	0.27000

8.3 Branched-chain Aminotransferase (BCAT; EC 2.6.1.42) Assay

Reference:

Peter Schadewalt (2000) Determination of Branched chain-L-amino acid aminotransferase activity, Methods in Enzymology, 324:23-32.

Microplate Assay Medium Chemicals

BAL Buffer, pH 8.3 with NAOH

KIC (ketoisocaproic acid) 42mM

KMV (ketomethyl valeric acid) 42mM

KIV (keto isovaleric acid) 42mM

Enzyme Dissolution Buffer 100mM

(Sodium phosphate buffer, pH 7.4)

Alanine amino transferase (ALAT)

Lactate dehydrogenase (LDH)

Nicotinamide adenine dinucleotide hydrogen (NADH)

Microplate Assay

1. Porcine mitochondria are prepared by initially weighing 20 g of respective tissue.
2. Homogenize tissue in 0.3M mannitol/1mM EDTA. Differential centrifugation steps are followed.
3. 800 x g x 5min at 4° C ; Decant supernatant and save. Discard pellet.

4. Supernatent at 6800 x g x 10 min at 4° C; Decant supernatant and discard. Resuspend pellet in 0.3M mannitol/1mM EDTA.
5. Resuspended pellet at 18000 x g x 10 min at 4° C; Decant supernatant and discard. Resuspend pellet (mitochiondria) in 0.3 M mannitol and determine protein concentration by BCA (bicincchoninic acid) method (Sigma-BCA1).
6. Solubilize mitochondria in 300mM mannitol containing 0.2% (v/v) Nonidet P-40 to a final concentration of 3.0 mg/mL protein. Keep on ice until use.
7. Combine BAL buffer (70µL), NADH (20µL), PLP (20µL), LDH (20µL) to make 130µL/well. Keep the working buffer on ice until added to microplate. This is the **working assay buffer**.
8. Add dd H_2O (30µL)/well.
9. Fill out microplate with α-KIC/KMV/KIV (20µL)/well for Δ substrate.
10. Mix 150µL **working assay buffer** with 100 µL solubilized mitochondria per well
11. Add 20 µL of ALAT to each well using a multi-channel pipette.
12. Measure disappearance of NADH at an absorbance of 340nm for 10-15 min with spectrophotometer set at 25 °C.

MICROPLATE ASSAY IN BRIEF (Each well)

20 µL α-KIC/KMV/KIV
30 µL dd H_2O
130µL **working assay buffer**
100 µL solubilized mitochondria
20 µL ALAT

300 µL Total volume

8.4 Branched-chain Amino Acid Oxidation Assay

Reference:

Miller RH and Harper AE (1988) Regulation of valine and α- ketoisocaproate metabolism in rat kidney mitochondria, Am J Physiol. 255:E475-481.

Oxidation Assay Medium Chemicals

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1. 148 mM mannitol
2. 48mM Sucrose
3. 79mM HEPES
4. 25mM KH_2PO_4
5. 2mM $MgCl_2$
6. 1mM EGTA
7. 8mM Na_2CO_3
8. 1.9mM NAD
9. 1.0mM Coenzyme A
10. 0.2mM Thiamine Pyro-Phosphate (TPP)
11. 3mM α-Ketoglutarate
12. 0.05mM Malate (sodium salt)

Oxidation Assay

1. Dissolve oxidation chemicals in ~300mls ddH_2O
2. Adjust pH to 7.4 (using NaOH)
3. Make up volume to 400mLs

4. Divide into 5 separate 75 mLs.
5. Add 1mM of ILE/LEU/VAL/KIC respectively (0.75 mL of 100mM conc. Stock)
6. Separate 46mLs of each solution into 50ml conical flask [**HOT media**]
7. Pour remaining 21 ml into 50 ml conical flask as well [**COLD Media**]
8. Add isotopes in the following order to HOT tubes.

230 µL of 1-^{14}C-Isoleucine

230 µL of 1-^{14}C-Leucine

230 µL of 1-^{14}C-Valine

230 µL of 1-^{14}C-KIC

(Isotope addition at 0.5 µCi/reaction)

OXIDATION ASSAY IN BRIEF (Each Vial)

2mL oxidation media

100µL intact mitochondria [5/10 mg protein/mL]

300µL Absorber [2:1, Ethylene Glycol Mono Methyl Ether : Ethanolamine]

- incubate at 30 °C for 4 hrs in a shaking water bath
- Stop reaction with 1mL 0.6M Sodium citrate(pH 3.0)
- Collect absorber receptacle 1hour after stopping of reaction in 7mL scintillation vial and add 5mL Atomlite and count radioactivity in scintillation counter.

8.5 Calculation of whole organ leucine oxidative capacity in gut and liver

Table 8.5.1 Mitochondrial yield, organ weights and leucine oxidative rates

Gut
Leucine Oxidation

0 Day

Mito Total	Wt of gut used	Avg gut weight	nmol oxdn/mg/hr	nmol/gut	**nmol/day**	**umol/day**
50	40	85	0.75	80	**1913**	**1.9**

3 Day

Mito Total	Wt of gut used	Avg gut weight	nmol oxdn/mg/hr	nmol/gut	**nmol/day**	**umol/day**
110	111	150	0.69	103	**2462**	**2.5**

7 Day

Mito Total	Wt of gut used	Avg gut weight	nmol oxdn/mg/hr	nmol/gut	**nmol/day**	**umol/day**
225	118.5	163.5	1.225	380	**9127**	**9.1**

Adult

Mito Total	Wt of gut used	Avg gut weight	nmol oxdn/mg/hr	nmol/gut	**nmol/day**	**umol/day**
225	118.5	6000	21.5	244937	**5878481**	**5878**

Liver
Leucine Oxidation

0 Day

Mito Total	Wt of liver used	Avg liver weight	nmol oxdn/mg/hr	nmol/liver	**nmol/day**	**umol/day**
234	20	42	44	21622	**518918**	**519**

3 Day

Mito Total	Wt of liver used	Avg liver weight	nmol oxdn/mg/hr	nmol/liver	**nmol/day**	**umol/day**
390	20	85	51.8	85859	**2060604**	**2060**

7 Day

Mito Total	Wt of liver used	Avg liver weight	nmol oxdn/mg/hr	nmol/liver	**nmol/day**	**umol/day**
880	20	95	27.5	114950	**2758800**	**2758**

Adult

Mito Total	Wt of liver used	Avg liver weight	nmol oxdn/mg/hr	nmol/liver	**nmol/day**	**umol/day**
750	20	2500	17.9	1678125	**40275000**	**40275**